Metabolically engineered cells for the production of polyunsaturated fatty acids

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(54) Title: METABOLICALLY ENGINEERED CELLS FOR THE PRODUCTION OF POLYUNSATURATED FATTY ACIDS

(57) Abstract: The present invention relates to the construction and engineering of cells, more particularly microorganisms for producing PUFAs with four or more double bonds from non-fatty acid substrates through heterologous expression of an oxygen requiring pathway. The invention especially involves improvement of the PUFA content in the host organism through fermentation optimization, e.g. decreasing the temperature and/or designing an optimal medium, or through improving the flux towards fatty acids by metabolic engineering, e.g. through over-expression of fatty acid synthases, over-expression of other enzymes involved in biosynthesis of the precursor for PUFAs, or codon optimization of the heterologous genes, or expression of heterologous enzymes involved in the biosynthesis of the precursor for PUFAs.

WO 2005/118814 A2

METABOLICALLY ENGINEERED CELLS FOR THE PRODUCTION OF POLYUNSATURATED FATTY ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Serial No. 60/577,245, filed on June 4, 2004.

Each of these applications, patents, and each document cited in this text, and each of the documents cited in each of these applications, patents, and documents ("application cited documents"), and each document referenced or cited in the application cited documents, either in the text or during the prosecution of the applications and patents thereof, as well as all arguments in support of patentability advanced during prosecution thereof, are hereby incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates generally to the production of fatty acids and particularly to the production of polyunsaturated fatty acids (PUFAs) in various cells, more specifically, to the expression of heterologous pathways in microorganisms for the production of fatty acids and particularly polyunsaturated fatty acids.

BACKGROUND OF THE INVENTION

PUFAs are polyunsaturated fatty acids with a long hydrocarbon chain composed of 18 or more carbon atoms having two or more double bonds and a terminal carboxylate group.

The properties of polyunsaturated fatty acids are highly influenced by the position of the double bond, and one differentiates omega-3 PUFAs, which have the first double bond at the third position counting from the methyl end of the carbon chain, and omega-6 PUFAs, which have the first double bond at the sixth position counting from the methyl end of the carbon chain. Eicosapentaenoic acid belongs to the former group, particularly eicosapentaenoic acid with double bonds in position 5, 8, 11, 14 and 17 (EPA) and docosahexaenoic acid, particularly docosahexaenoic acid with double bonds in position 4,7,10, 13,16, 19 (DHA), while, for example, arachidonic acid (ARA) belongs to the latter group.

PUFAs are essential for humans, and it has been proven that they have many beneficial effects on human health, including proper development of brain and visual functions and prevention of disease, such as cardiovascular disease and cancer.

The omega-6 PUFA arachidonic acid plays an important role in the structure and function of biological membranes, and is a precursor of the biologically active prostaglandins and leukotrienes. Arachidonic acid is necessary for the neurological and neurophysiological development of both term and preterm infants, and many expert organizations, including the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) recommend that infant formula should be supplemented with arachidonic acid.

The omega-3 PUFA EPA and DHA, as well, possess a number of physiological functions in humans. They are part of the human tissue, and in the rod outer segment in the retina, DHA represents more than 60% of the total fatty acids. DHA is regarded to be essential for proper visual function and neurological development of infants. Preterm and young infants are unable to synthesize sufficient amounts of DHA and receive the remaining by breast milk. DHA also reduces or eliminates the risk factor involved in various diseases like cardiovascular diseases and exerts positive effects on hypertension, arthritis, arteriosclerosis and thrombosis.

Various publications, patents and patent application focus on the production of PUFA from fatty acid substrates. Recently, the pathway from linoleic acid to arachidonic acid was reconstituted in *S. cerevisiae* (Domergue et al., 2003, Beaudoin et al., 2000) and synthesis of polyunsaturated fatty acids was established by supplying the precursor metabolite, linoleic acid, to the medium.

Other groups have established parts of the PUFA pathway in reconstitution experiments. For example, US 6,432,684 describes the sequence of a delta-5 desaturase from human, which, when expressed in yeast, produces arachidonic acid, when dihomo-gamma-linolenic acid is supplied.

US 2002/0170090 describes an omega-3 desaturase from *Caenorhabditis elegans* and its expression in various organisms including bacteria, cyanobacteria, phytoplanton, algae, fungi, plants and animals, and the production of a lipid from an organism that expresses

the omega-3 desaturase. The enzyme catalyzes the conversion of omega-6 fatty acids with 18, 20 and 22 carbon atoms to the corresponding omega-3 fatty acids. Yeast cells, expressing the omega-3 desaturase from *C. elegans*, converted exogenously supplied linoleic acid and omega-6 docosatetraenoic acid into alpha-linolenic acid and omega-3 docosapentaenoic acid, respectively.

PCT/US98/07422 describes the isolation of a delta-5 desaturase from *Mortierella alpina* and expression of said enzyme in microbial cells, particularly in *Saccharomyces cerevisiae*, and reports production of arachidonic acid when dihomo-gamma-linolenic acid is supplied in the growth medium.

WO 99/27111 describes a delta-6 desaturase from *C. elegans* and its expression in yeast, which led to production of gamma-linolenic acid from exogenously, supplied oleic acid.

In WO99/33958, the expression of a delta-5 desaturase (originally obtained from *C. elegans)* in microorganisms, such as algae, bacteria and fungi, and particularly, its expression in yeast is disclosed.

WO 02/44320 describes a number of different human elongases, many of which have been tested for functionality in yeast using a number of different fatty acids as externally supplied substrates.

A method for the production of arachidonic acid in transgenic organisms (WO 03/012092) has been applied. Here, the inventors describe the expression of a delta-5 desaturase, which leads to the production of arachidonic acid in yeast; however, it requires dihomo-gamma linolenic acid as an external substrate.

The inventors of PCT/US98/07421 test the expression of various desaturases including delta-12 desaturase, delta-6 desaturase and delta-5 desaturase and reconstitute the function of these enzymes by adding fatty acid substrates to the growth medium and analysing their conversion.

In all the above-mentioned publications, patents and patent applications, processes have been described, where it is necessary to supply fatty acids as external substrates in the medium in order to produce PUFAs.

In the following a few publications are described, which report production of PUFAs with up to 3 double bonds from non-fatty acid substrates.

In US 6,355,861 it is shown that the expression of delta-12 and delta-6 desaturase from *Cynecosystis* in *Cynecococcus* leads both to the production of linoleic acid and gamma-linolenic acid, fatty acids with two double bonds and three double bounds, respectively. Furthermore, expression of a delta-6 desaturase from prime rose in a bacterial, fungal or plant cell is disclosed, including expression of said delta-6 desaturase in various plants for the production of gamma-linolenic acid.

US 6,136,574 describe the production of gamma-linoleic acid in yeast from endogenously available oleic acid.

PCT/US98/07126 describes the expression of a delta-6 desaturase and a delta-12 desaturase and reports, for example that expression of these genes in a host cell leads to the production of gamma-linoleic acid.

No prior art reference discloses successful heterologous PUFA production with four or more double bonds in microorganisms from carbon sources other than fatty acids, despite the fact that a high number of different genes involved in PUFA biosynthesis have been identified. This clearly demonstrates that it is a difficult task to produce PUFAs with four or more double bonds at sufficient or detectable titers in microorganisms that usually do not produce PUFA. Although the inventors of US 2003/0177508 describe sequences of four genes that are involved in PUFA elongation and show the function of all these genes, the inventors can only speculate in an example (example III) that expression of delta-12 desaturase, delta-6 desaturase, delta-5 desaturase, and a *Mortierella alpina* elongase cDNA in yeast could result in the production of arachidonic acid without the need of exogenous supply of fatty acids.

As an intermediate summary of the above paragraphs, it can be concluded that, except for the speculations specified in US 2003/0177508, there have until now been no reports on expressing a heterologous pathway for the production and PUFA from non-fatty acid substrates in microorganisms. Until now, reports concerning heterologous PUFA production from non-fatty acid substrates in microscopic hosts, such as yeast, have been limited to PUFAs with less than four double bonds, namely three or less double bonds, such as linoleic acid and gamma-linolenic acid.

If the strategy suggested in US 2003/0177508 is followed, one will expect a low content of arachidonic acid in bakers yeast, as this organism has a low content (approximately 10% of cell dry weight) of fatty acids. Furthermore, the fatty acids in bakers yeast primarily consists of fatty acids with 16 carbon atoms, and the most dominant mono-unsaturated fatty acid is palmitoleic acid, which can not serve as a precursor for synthesis of arachidonic acid. The result of simply expressing the mentioned four genes in *S. cerevisiae,* where expression of these four genes, results in an arachidonic acid content of 0.8% of the fatty acids, or corresponding to less than 0.08% of the yeast dry weight.

The production of polyunsaturated omega-3 and omega-6 fatty acids with four and five double bonds, but not six double bonds, has recently been reported in plants. Qi and co-workers were able to produce the omega-3 fatty acid EPA and the omega-6 fatty acid arachidonic acid in the plant *Arabidopsis thaliana* (Qi et al. 2004).

Qi and co-workers show for the first time that arachidonic acid and EPA can be produced via a heterologous pathway in an organism, such as a plant, using a non-fatty acid substrate. The authors succeed by simultaneously expressing genes coding for delta-9 elongase, delta-8 desaturase and delta-5 desaturase, an approach that makes use of the endogenous delta-12 desaturase and endogenous omega-3 desaturase activities of *A. thaliana* for production of arachidonic acid and EPA. In many organisms, including microorganisms, such as many yeasts and filamentous fungi, it would be necessary to express at least 4 or at least 5 heterologous genes in order to produce PUFA with at least four or at least five double bonds. Until now the expression of more than 3 heterologous genes at the same time for the production of PUFAs has never been applied. Moreover, the production of polyunsaturated fatty acids from non-fatty acid substrates has not yet been shown in non-plant cells.
In WO 2004/057001 the inventors describe that the technology works in both plants and microorganims. However, the inventors have yet only confirmed the described technology in plants.

PCT/US2004/014541 describes the production of PUFAs, such as arachidonic acid and EPA using oleaginous yeast. The inventors define oleaginous yeast as yeast that can accumulate at least 25% of its cell dry weight as oil. The invention uses oleaginous yeast such as *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon*

and *Lipomyces* as host cell. It does not provide or claim information about other organisms or non-oleaginous yeast such as *Saccharomyces cerevisiae*. The inventors exemplify their technology by heterologous expression of three additional enzymes in *Yarrowia lipolytica* using delta-6 desaturase, delta-5 desaturase and delta-17 desaturase. The latter is equivalent to omega-3 desaturases. This approach makes use of the endogenous delta-12 desaturase.

In WO2005/01236 the inventors show that it is possible to produce PUFAs in yeast by supplying a fatty acid together with a non-fatty acid substrate. The inventors express delta-4 desaturase, elongases and/or delta-5 desaturase in *Saccharomyces cerevisiae*. By providing EPA or stearidonic acid together with galactose, *Saccharomyces cerevisiae* produces DHA.

PUFAs are increasingly supplied in food, for example in infant formula, and also in pharmaceutical formulations. A general source of PUFAs is fish oil. However, the fatty acid content of fish oil may vary during the fishing season and in some cases the fish oil may be contaminated because of environmental pollution. Besides this, fish oil has an obnoxious smell, which precludes its use as a food supplement.

Hence, proper and expensive purification steps are necessary for some application of PUFAs. The need for PUFAs produced by well-defined methods and in large quantities will increase dramatically during the next 5-10 years, and it is estimated that PUFAs will be used in many different products as a supplement. In order to meet the increasing demand for high quality PUFAs focus has moved towards reproducible production methods and this includes production methods using non-fatty acids substrates. The latter allows a more defined production of unsaturated fatty acids.

The present invention addresses this demand, and presents an efficient new, cost effective and alternative method for the high-level production of mono unsaturated fatty acids and particular PUFAs.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to the construction and engineering of non-plants more particularly microorganisms for producing PUFAs with four or more double

bonds from non-fatty acid substrates through heterologous expression of an oxygen requiring pathway.

In another aspect, the present invention refers to the construction and engineering of non-plants more particularly microorganisms for producing PUFAs with four or more double bonds using a non-fatty acid substrate or substrates as one or several exclusive carbon sources through heterologous expression of an oxygen requiring pathway.

In particular, the present invention describes a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen-requiring pathway in a *Saccharomyces cerevisiae* grown on a non-fatty acid substrate.

Furthermore, the present invention relates to the construction and engineering of microorganisms for heterologous production of mono unsaturated fatty acid and particular PUFAs, including oleic acid, linoleic acid, alpha-linolenic, gamma-linoleic acid, dihomo-gamma-linolenic acid, arachidonic acid, 5,8,11,14,17-eicosapentaenoic acid (EPA), docosatetraenoic acid, stearidonic acid, eicosadienoic aicd, eicosatrienoic acid, eicosatetraenoic acid, 7,10,13,16,19-docosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid (DHA).

The invention especially involves a genetically transformed microorganism containing a heterologous pathway from stearic acid to mono unsaturated fatty acids and PUFAs, i.e. oleic acid, arachidonic acid, DHA or EPA through expression of the following heterologous enzymes delta-9 desaturase, delta-12 desaturase, delta-9 elongase, delta-8 desaturase, omega-3 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, delta-5 elongase, delta-4 desaturase or subsets hereof (Figure 1 and Figure 2).

Furthermore, the present invention relates to improvement of the PUFA content in the host organism through optimization of fermentation conditions, e.g. decreasing the temperature and/or designing an optimal medium, or through improving the flux towards fatty acids by metabolic engineering, e.g. through overexpression of fatty acid synthases, over-expression of other enzymes involved in biosynthesis of the precursors for PUFAs, or codon optimization of the heterologous genes, or expression of heterologous enzymes involved in the biosynthesis of the precursor for PUFAs, i.e. oleic acid.

The invention also relates to a composition comprising at least 2 % polyunsaturated fatty acids produced from a microorganism that expresses a heterologous pathway leading to PUFAs.

These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

DETAILED DESCRIPTION OF THE INVENTION

It should be understood that any feature and/or aspect discussed above in connection with the methods according to the invention apply by analogy to the uses.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

As will be apparent, preferred features and characteristics of one aspect of the invention may be applicable to other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The present inventors have developed a novel, alternate and highly cost effective method for producing a polyunsaturated fatty acid by construction and engineering of non-plant host cells, especially microorganisms, for producing PUFAs with four or more double bonds from non-fatty acid substrates through heterologous expression of an oxygen requiring pathway.

The invention relates to the construction and engineering of such non-plant host cells for heterologous production of mono unsaturated fatty acids and PUFAs, including oleic acid, linoleic acid, alpha-linolenic, gamma-linoleic acid, dihomo-gamma-linolenic acid, arachidonic acid, 5,8,11,14,17-eicosapentaenoic acid (EPA), docosatetraenoic acid, stearidonic acid, eicosatetraenoic acid, 7,10,13,16,19-docosapentaenoic acid and 4,7,10,13,16,19-docosahexaeonic acid (DHA).

The invention involves genetically modified non-plant host cells, especially microorganisms, containing a heterologous pathway from stearic acid to mono unsaturated fatty acids and PUFAs, e.g. oleic acid, arachidonic acid, DHA or EPA through expression of heterologous genes encoding the following enzymes delta-9 desaturase, delta-12 desaturase, delta-9 elongase, delta-8 desaturase, omega-3 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, delta-5 elongase, delta-4 desaturase or subsets hereof (Figure 1 and Figure 2).

Thus, in one aspect, the present invention provides a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a non-plant host cell grown on a non-fatty acid substrate.

In a particular preferred embodiment said non-fatty acid substrate is the exclusive carbon source.

Polyunsaturated fatty acid

In the present context the term "polyunsaturated fatty acid" relates to a long hydrocarbon chain composed of 18 or more carbon atoms having at least 4 double bonds and a terminal carboxylate group.

In a preferred embodiment the polyunsaturated fatty acid produced by the method of the present invention relates to polyunsaturated fatty acids with at least 4 double bonds, such as 4 double bonds, 5 double bonds or 6 double bonds.

As the skilled artisan would recognise, a fatty acid may be esterified to form triglycerides and/or phospholipids as well as sphingolipids. Thus, in one embodiment the present invention also relates to such esterified products.

Furthermore, the fatty acid product of the present invention can be free fatty acids. Free fatty acids have a free carboxyl group, are not chemically connected to any other compound including triacylglycerides, phospholipids or sphingolipids, and can be present freely in any compartment of the cell.

Heterologous expression

By "expression", it is meant the production of a functional polypeptide through the transcription of a nucleic acid segment into mRNA and translation of the mRNA into a protein.

By "heterologous expression", it is generally meant that a nucleic acid, not naturally present in the host genome, is present in the host cell and is operably linked to promoter and terminator nucleic acid sequences in a way so it is expressed in the host cell.

Also, in the present context heterologous expression further relates to the presence of a nucleic acid with a similar function to a naturally present nucleic acid, wherein the expression of said heterologous nucleic acid product changes the fatty acid composition. For example, expression in yeast of a fungal delta-9 desaturase with different substrate specificity than the native yeast delta-9 desaturase changes the fatty acid composition of yeast (Example 36).

Said nucleic acid may be contained on an extrachromosomal nucleic acid construct or may be integrated in the host genome. Methods for isolation of nucleic acids for heterologous expression and preferred embodiments of heterologous expression are further described in details below.

By heterologous expression of a pathway is meant that several genes are expressed heterologously, whose gene products constitute steps in a pathway, not naturally present in the host.

Oxygen requiring pathway

An oxygen-requiring pathway shall mean that at least one of the enzymes in the pathway requires oxygen to function. For example the expression of nucleic acids coding for desaturase usually leads to a pathway that requires oxygen for activity as desaturases usually are oxygen-requiring enzymes.

Non fatty acid substrate

In the present context, a "non-fatty acid substrate" relates to any substrate, but not a fatty acid, with two or more carbon atoms, such as but not limited to sugars, such as

glucose, mannose, fructose, sucrose, galactose, lactose, erythrose, threose, ribose, glyceraldehyde, dihydroxyacetone, ribulose, cellobiose, starch, glycogen, trehalose, maltose, maltotriose, xylose, arabinose, stachyose, raffinose, or non-fermentable carbon source, such as but not limited to ethanol, lactate, acetate and glycerol.

Exclusive carbon source or exclusive carbon sources

Usually, a living organism needs a supply of many or all of the macroelements such as carbon, sulphur, phosphor, nitrogen, oxygen or hydrogen. An organism may grow on mixtures of different carbon sources, such as a fatty acid substrate and a non-fatty acid substrate. If a substrate or substrates is refered to an exclusive carbon or exclusive carbon sources, it is only that substrate or those substrates that is supplied to the organism as a carbon source. This shall not exclude other macroelements or other nutrianol requirements, such as requirements for example for trace elements and vitamins. For example, if a non-fatty acid substrate is exclusively supplied as a carbon source. This means, it is only that non-fatty acid that is supplied as a carbon source without supplying another carbon source. However, this does not exclude other macroelements or other nutrional requirements.

Non-plant host cell

In the present context the term "non-plant host cell" relates to host cells selected from the group consisting of micro-organisms, animals, fungi, bacteria, invertea (insects) or protozoa. In particular, it relates to microscopic organisms, including bacteria, unicellular algae, protozoans and microscopic fungi, including yeast.

More specifically, the microorganism may be a fungus, and more specifically a filamentous fungus belonging to the genus of *Aspergillus*, e.g. *A. niger*, *A. awamori*, *A. oryzae*, *A. nidulans*, a yeast belong to the genus of *Saccharomyces*, e.g. *S. cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum* , a yeast belonging to the genus *Kluyveromyces, e.g. K. lactis K. marxianus var. marxianus, K. thermotolerans*, a yeast belonging to the genus *Candida, e.g. C. utilis, C. tropicalis, C.albicans, C. lipolytica, C. versatilis,* a yeast belonging to the genus *Pichia, e.g. P. stipidis, P. pastoris, P. sorbitophila,* or other yeast genus, *e.g. Cryptococcus, Debaromyces, Hansenula, Pichia, Yarrowia, Zygosaccharomyces* or *Schizosaccharomyces*, but are not limited to these examples. Concerning other microorganisms a non-exhaustive list of suitable filamentous

fungi is supplied: a species belonging to the genus *Penicillium*, *Rhizopus*, *Fusarium, Fusidium, Gibberella, Mucor, Mortierella, Trichoderma*, among others.

Concerning bacteria a non-exhaustive list of suitable bacteria is given as follows: a species belonging to *Bacillus*, a species belonging to the genus *Escherichia*, a species belonging to the genus *Lactobacillus*, a species belonging to the genus *Corynebacterium*, a species belonging to the genus *Acetobacter,* a species belonging to the genus *Acinetobacter,* a species belonging to the genus *Pseudomonas*, etc.

The preferred microorganisms of the invention may be *S. cerevisiae, A. niger, Escherichia coli or Bacillus subtilis.*

In a presently preferred embodiment the preferred microorganisms of the invention is *S. cerevisiae* for a number of reasons. *S.cerevisiae* is a well know model organism, and has undergone tremendous research for thousands of years, its physiology is well understood, and analytical tools are available to invenstigate the metabolism at any level, such as the genome level, the transcript level, the protein level, the metabolite level and the flux level. Hence this allows rapidly the development of metabolic engineering strategies and therefore the identification of efficient genetic engineering targets in order to improve the PUFA yield and production rates. Besides this, Saccharomyces cerevisiae has GRAS status, and fermentation technology is well established.

The constructed and engineered microorganism can be cultivated using commonly known processes, including chemostat, batch, fed-batch cultivations, etc.

In a specific aspect, the invention relates to a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a non-plant host cell grown on a non-fatty acid substrate, with the proviso that said method does not comprise combining the heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase in a host cell.

In a preferred embodiment, a method for producing polyunsaturated fatty acids with four or more double bonds is provided comprising heterologous expression of an oxygen requiring pathway in a non-plant host cell grown on a non-fatty acid substrate, wherein said heterologous expression increases the content of each individual specific

polyunsaturated fatty acid of particularly ARA, EPA and DHA to more than 2 % of the total fatty acid content. The content of intermediate PUFAs on the biosynthetic pathway towards ARA, EPA or DHA can be more than 2 % but does not need to be more than 2 %.

The increase of PUFA content is described in more details below.

Heterologous expression of at least 4 specific genes

One aspect of the present invention relates to simultaneous heterologous expression of at least 4 specific genes for production of PUFAs.

Thus, in one embodiment the present invention relates to a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a *Saccharomyces cerevisiae* grown on a non-fatty acid substrate, wherein the heterologous expression comprises combining heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase.

In another embodiment the present invention relates to a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a *Saccharomyces cerevisiae* grown on a non-fatty acid substrate, wherein the heterologous expression comprises combining heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase.

Specifically, one embodiment describes a method for producing a polyunsaturated fatty acid comprising the steps of

(a) isolating at least 4 nucleotide sequences, each having identity of at least 75% to one of the nucleotide sequence selected from the group consisting of SEQ ID NO: 1-38;

(b) constructing a vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a);

(d) exposing said host cell to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product.

and obtaining said polyunsaturated fatty acid.

More specifically, one embodiment describes a method for producing a polyunsaturated fatty acid comprising the steps of

(a) isolating at least 4 nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase

(b) constructing one or more vectors comprising said isolated nucleotide sequences of step (a) and/or integrating said isolated nucleotide sequences into the genome of *Saccharomyces cerevisiae*;

(c) optionally, transforming said vector(s) of step (b) into a *Saccharomyces cerevisiae* for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a);

(d) growing said *Saccharomyces cerevisiae* on a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product

and obtaining said polyunsaturated fatty acid.

Another embodiment describes a method for producing a polyunsaturated fatty acid comprising the steps of

(a) isolating at least 4 nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase

(b) constructing one or more vectors comprising said isolated nucleotide sequences of step (a) and/or integrating said isolated nucleotide sequences into the genome of *Saccharomyces cerevisiae*;

(c) optionally, transforming said vector(s) of step (b) into a *Saccharomyces cerevisiae* for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a);

(d) growing said *Saccharomyces cerevisiae* on a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product

and obtaining said polyunsaturated fatty acid.

As mentioned elsewhere, the heterologous expression may further comprise heterologous expression of a nucleotide sequence encoding delta-9 desaturase, delta-5 elongase, omega-3 desaturase, and/or delta-4 desaturase.

Specifically, wherein SEQ ID NO: 1-4 encode delta-9 desaturases, wherein SEQ ID NO: 5-10, 93, 95, 113 encode delta-12 desaturases, wherein SEQ ID NO: 11- 15, 97, 99 encode delta-6 desaturases, wherein SEQ ID NO: 16-21, 101, 103 encode delta-6 elongases, wherein SEQ ID NO: 22-27, 105, 107 encode delta-5 desaturases, wherein SEQ ID NO: 30-34, 87, 89, 111 encode omega-3 desaturases, wherein SEQ ID NO: 19, SEQ ID NO: 19, 28-29, 101 encode delta-5 elongases wherein SEQ ID NO: 35-36, 109 encode delta-4 desaturases, wherein SEQ ID NO: 37 encode delta-9 elongase and wherein SEQ ID NO: 38 encode delta-8 desaturase.

In one embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase in a host cell.

In another embodiment the invention relates to a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a *Saccharomyces cerevisiae* host cell grown on exclusively non-fatty acid substrates as carbon sources, which is the exclusive carbon source.

The expression of said four genes allows the production of arachidonic acid and/or one or more of its intermediate precursors in host cells that endogenously only produce fatty acids of up to 18 carbon atoms of length with up to one double bond.

Furthermore, expression of said pathway generally improves the production of arachidonic acid in a host cell and can also lead to improved production of one or more of its intermediate precursors. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In another embodiment of the present invention, a method for producing a polyunsaturated fatty acid is provided, comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase in a host cell.

The expression of said four genes allows the production of arachidonic acid and or one or more of its intermediate precursors in host cells that endogenously produces only fatty acids of up to 18 carbon atoms of length with up to one double bond.

Besides this, expression of said pathway generally improves the production of arachidonic acid and/or one or more of its intermediate precursors in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived for example from plants, animals or microorganisms, can also be used.

In a third embodiment, the present invention relates to a method for producing polyunsaturated fatty acids comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase and delta-5 elongase in a host cell.

The expression of said five genes allows the production of docosatetraenoic acid, more specifically omega-6 docosatetraenoic acid and/or one or one more of its intermediate precursors in host cells that endogenously produce only fatty acids of up to 18 carbon atoms of length with up to one double bond.

Furthermore, expression of said pathway generally improves the production of omega-6 docosatetraenoic acid in a host cell and can also lead to improved production of one or more of its intermediate precursors. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In another embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, delta-5 desaturase and delta-5 elongase in a host cell.

The expression of said five genes allows the production of docosatetraenoic acid, more specifically omega-6 docosatetraenoic acid and or one or more of its intermediate precursors in host cells that endogenously produce only fatty acids of up to 18 carbon atoms of length with up to one double bond.

Furthermore, expression of said pathway generally improves the production of omega-6 docosatetraenoic acid in a host cell and can also lead to improved production of one or more of its intermediate precursors. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In a further embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase and omega-3 desaturase in a host cell.

The expression of said five genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in host cells that endogenously only produce fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, the expression of said five genes allows the production of eicosapentaenoic acid in said host cells.

Furthermore, the expression of said five genes generally improves the production of eicosapentaenoic acid and/or one or more of its intermediate precursors, including arachidonic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In yet another embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase and omega-3 desaturase in a host cell.

The expression of said five genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in host cells that endogenously produce only fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, the expression of said five genes allows the production of eicosapentaenoic acid in said host cells.

Furthermore, the expression of said five genes generally improves production of eicosapentaenoic acid and/or one or more of its intermediate precursors, including arachidonic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In a further embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, omega-3 desaturase and delta-5 elongase in a host cell.

The expression of said six genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in host cells that endogenously only produce fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, it allows the production of docosapentaenoic acid in said host cells.

Furthermore, the expression of said six genes generally improves production of docosapentaenoic acid and/or one or more of its intermediate precursors, including docosatetraenoic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In another embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, delta-5 desaturase omega-3 desaturase and delta-5 elongase in a host cell.

The expression of said six genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in host cells that endogenously produce only fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, it allows the production of docosapentaenoic acid in said host cells.

Furthermore, the expression of said six genes generally improves production of docosapentaenoic acid and/or one or more of its intermediate precursors, including docosatetraenoic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In another embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, omega-3 desaturase, delta-5 elongase and delta-4 desaturase in a host cell.

The expression of said seven genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in host cells that endogenously produce only fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, it allows the production of docosahexaenoic acid in said host cells.

Furthermore, the expression of said seven genes generally improves the production of docosahexanenoic acid and/or one or more of its intermediate precursors, including docosatetraenoic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

In another embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining the heterologous expression of genes encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, delta-5 desaturase omega-3 desaturase, delta-5 elongase and delta-4 desaturase in a host cell.

The expression of said seven genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in a host that endogenously produces only fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, it allows the production of docosahexaenoic acid in said host cells.

Furthermore, the expression of said seven genes generally improves production of docosahexanenoic acid and/or one or more of its intermediate precursors, including docosatetraenoic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived for example from plants, animals or microorganisms, can also be used.

In another preferred embodiment, a method according to the present invention is provided, wherein any one of the different combinations of heterologous expression described above further comprises heterologous expression of a gene encoding a delta-9 desaturase that preferentially uses stearic acid as substrate. Expression of said gene encoding a stearic acid specific delta-9 desaturase allows a shift in the fatty acid composition from palmitoleic acid towards oleic acid as compared to an unmodified host cell. Expression of said gene in combination with one of the pathways described above for production of polyunsaturated fatty acids therefore further improves the production of polyunsaturated fatty acids.

In a particularly preferred embodiment, the present invention relates to a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a *Saccharomyces cerevisiae* host cell grown on a non-fatty acid substrate or non-fatty acid substrates which is/are the exclusive carbon sources, wherein the combined heterologous expression consists of heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase.

In another particular preferred embodiment, the present invention relates to a method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a *Saccharomyces cerevisiae* host cell grown on a non-fatty acid substrate which is the exclusive carbon source, wherein the combined heterologous expression consists of heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase.

A further particular preferred embodiment relates to a method according to the present invention, wherein the combined heterologous expression further comprises heterologous expression of a nucleotide sequence encoding delta-5 elongase, omega-3 desaturase, and/or delta-4 desaturase.

Another further particular preferred embodiment relates to a method according to the present invention, wherein said combined heterologous expression further comprises heterologous expression of a nucleotide sequence encoding a delta-9 desaturase.

Substrate

The fermentation substrate for the production of PUFAs according to the present aspect maybe any complex medium or defined medium e.g. containing sugars, such as glucose, mannose, fructose, sucrose, galactose, lactose, erythrose, threose, ribose, glyceraldehyde, dihydroxyacetone, ribulose, cellobiose, starch, glycogen, trehalose, maltose, maltotriose, xylose, arabinose, stachyose, raffinose, or non-fermentable carbon sources, such as ethanol, acetate, lactate, or glycerol, or oils, such as oils derived from plants, animals or microorganisms or fatty acids, such as butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic

acid, palmitoleic acid, oleic acid, elaidic acid, *cis*-vaccenic acid, linoleic acid, alpha-linolenic, gamma-linoleic acids, dihomo-gamma-linolenic acid, arachidonic acid, EPA, 7,10,13,16,19-docosapentaenoic acid and 4,7,10,13,16,19-docosahexaeonic acid DHA.

Host cell

In the present context the term "host cell" relates to host cells selected from the group consisting of micro-organisms, animals, fungi, bacteria, invertea (insects), plants or protozoa. In particular, it relates to microscopic organisms, including bacteria, viruses, unicellular algae, protozoans and microscopic fungi including yeast.

In a presently preferred embodiment the host cell is a non-plant host cell as described above.

More specifically, the microorganism may be a fungus, and more specifically a filamentous fungus belonging to the genus of *Aspergillus*, e.g. *A. niger*, *A. awamori*, *A. oryzae*, *A. nidulans*, a yeast belong to the genus of *Saccharomyces*, e.g. *S. cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum,* a yeast belonging to the genus *Kluyveromyces, e.g. K. lactis K. marxianus var. marxianus, K. thermotolerans*, a yeast belonging to the genus *Candida, e.g. C. utilis, C. tropicalis, C.albicans, C. lipolytica, C. versatilis,* a yeast belonging to the genus *Pichia, e.g. P. stipidis, P. pastoris, P. sorbitophila,* or other yeast genus*, e.g. Cryptococcus, Debaromyces, Hansenula, Pichia, Yarrowia, Zygosaccharomyces* or *Schizosaccharomyces*. Concerning other microorganisms a non-exhaustive list of suitable filamentous fungi is supplied: a species belonging to the genus *Penicillium*, *Rhizopus*, *Fusarium, Fusidium, Gibberella, Mucor, Mortierella, Trichoderma*, among others

Concerning bacteria a non-exhaustive list of suitable bacteria is given as follows: a species belonging to *Bacillus*, a species belonging to the genus *Escherichia*, a species belonging to the genus *Lactobacillus*, a species belonging to the genus *Corynebacterium*, a species belonging to the genus *Acetobacter,* a species belonging to the genus *Acinetobacter,* a species belonging to the genus *Pseudomonas*, etc., that are well known in the art.

The preferred microorganisms of the invention may be *S. cerevisiae, A. niger, Escherichia coli or Bacillus subtilis.*

The constructed and engineered microorganism can be cultivated using commonly known processes, including chemostat, batch, fed-batch cultivations, etc.

Thus, in one preferred embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining heterologous expression of genes encoding various desaturases and elongases in a host cell as described herein, wherein said host cell is selected from the group consisting of plants, micro-organisms, animals, fungi, bacteria, invertea (insects) or protozoa.

In a particular preferred embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining heterologous expression of genes encoding various desaturases and elongases in a host cell as described herein, wherein said host cell is a fungus, and preferably, wherein said fungus is a filamentous fungus or a yeast.

In one embodiment said yeast is selected from the group of the genus *Saccharomyces, Kluyveromyces, Candida, Pichia, Cryptococcus, Debaromyces, Hansenula, Yarrowia, Zygosaccharomyces Schizosaccharomyces, Lipomyces*.

In a preferred embodiment said yeast is *Saccharomyces cerevisiae.*

In another embodiment said filamentous fungus is selected from the group of the genus *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium, Fusidium, Gibberella, Mucor, Mortierella or Trichoderma*.

In further embodiment said *Aspergillus* is selected from the species *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus oryzae* or *Aspergillus nidulans.* And in a presently most preferred embodiment, said host is *Aspergillus niger.*

In another preferred embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining heterologous expression of genes encoding various desaturases and elongases in a host cell as described i herein, wherein said host is a bacterium.

In one embodiment, said bacterium is selected from the group of *Bacillus*, *Escherichia*, *Lactobacillus*, *Corynebacterium*, *Acetobacter, Acinetobacter, or* Pseudomonas

In a presently most preferred embodiment said bacterium is Bacillus subtilis.

In another presently most preferred embodiment said one host cell is Escherichia coli.

In a presently preferred embodiment, the present invention relates to a genetically modified *Saccharomyces cerevisiae* capable of producing polyunsaturated fatty acids with four or more double bonds when grown on a non-fatty acid substrate.

In a presently most preferred embodiment, the present invention relates to a genetically modified *Saccharomyces cerevisiae* according to the invention, wherein said *Saccharomyces cerevisiae* is capable of producing polyunsaturated fatty acids with four or more double bonds when grown on a non-fatty acid substrate as the exclusive carbon source.

Polyunsaturated fatty acid

In the context of the present invention, a polyunsaturated fatty acid relates to a chemical compound with a long hydrocarbon chain composed of 18 or more carbon atoms having at least 4 double bonds and a terminal carboxylate group, having at least 5 double bonds and a terminal carboxylate group or having 6 double bonds and a terminal carboxylate group.

When applying the specific heterologous genes described above several intermediate products may be formed, and thus such intermediate products are included in the present invention. However, in some or many cases some or all of the intermediate products may be present at low levels that may not be easy to detect.

In the present context these intermediate products could be oleic acid, linoleic acid, gamma-linolenic acid, dihomo-gamma-linoleic acid, eicosadienoic acid, particularly, eicosadienoic acid with double bonds in position 11 and 14, eicosatrienoic acid, particularly, eicosatrienoic acid with double bonds in position 11, 14, and 17, arachidonic acid, docosatetraenoic acid, particularly docosatetraenoic acid with double bonds at position 7, 10, 13, 16, alpha-linoleic acid, stearidonic acid, eicosatetraenoic acid,

particularly eicosatetraenoic acid with double bonds in position 8, 11, 14 and 17, eicosapentaenoic acid, particularly eicosaoentaenoic acid with double bonds in position 5, 8, 11, 14 and 17 or docosapentaenoic acid.

In one preferred embodiment, a method according to the present invention is provided, wherein the polyunsaturated fatty acid comprises at least 4 double bonds, such as 4 double bonds, such as 5 double bonds and such as 6 double bonds.

In another preferred embodiment, said polyunsaturated fatty acid is produced from a non-fatty acid substrate.

In one preferred embodiment, said polyunsaturated fatty acid is produced from a fatty acid substrate with less than 4 double bonds in host cells originally devoid of endogenous expression of at least one of the enzymes selected from the group consisting of delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, omega-3 desaturase, delta-5 elongase and delta-4 desaturase.

In another preferred embodiment the polyunsaturated fatty acid is selected from the group consisting of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.

In one preferred embodiment, said polyunsaturated fatty acid is arachidonic acid.

Specifically, a method for producing arachidonic acid is provided comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, 99 isolating a third nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 16-21, 101, 103 and isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107.

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product.

and obtaining said arachidonic acid.

Or, alternatively, the present invention relates to a method for producing arachidonic acid comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to the nucleotide sequence set forth in SEQ ID NO: 37, isolating a third nucleotide sequence having at least 75% identity to the nucleotide sequence set forth in SEQ ID NO: 38 and isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product.

and obtaining said arachidonic acid.

In another preferred embodiment, said polyunsaturated fatty acid is eicosapentaenoic acid.

Specifically, a method for producing eicosapentaenoic acid is provided comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, 99 isolating a third nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 16-21, 101, 103 isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107 and isolating a fifth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89, 111;

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product.

and obtaining said eicosapentaenoic acid.

Or, alternatively, a method for producing eicosapentaenoic acid in provided comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to the nucleotide sequence set forth in SEQ ID NO: 37, isolating a third nucleotide sequence having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 38, isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107 and

isolating a fifth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89, 111;

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product.

and obtaining said eicosapentaenoic acid.

In one preferred embodiment, said polyunsaturated fatty acid is docosahexanoic acid.

Specifically, a method for producing docosahexaenoic acid is provided comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, 99 isolating a third nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 16-21, 101, 103 isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107 isolating a fifth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89, 111 isolating a sixth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 19, 28, 29, 101 and isolating a seventh nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 35-36, 109;

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product, and

obtaining said docosahexaenoic acid.

Or, the present invention also relates to a method for producing docosahexaenoic acid comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to the nucleotide sequence set forth in SEQ ID NO: 37, isolating a third nucleotide sequence having at least 75% identity the nucleotide sequence set forth in SEQ ID NO: 38, isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99 105, 107 isolating a fifth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89, 111 isolating a sixth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 19, 28, 29, 101 and isolating a seventh nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 35-36, 109;

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product, and

obtaining said docosahexaenoic acid.

In yet another preferred embodiment, said polyunsaturated fatty acid is docosapentaenoic acid.

Specifically, a method for producing docosapentaenoic acid is provided comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, 99 isolating a third nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 16-21, 101, 103 isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107 isolating a fifth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 19, 28, 29, 101 and isolating a sixth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89, 111;

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product, and

obtaining said docosapentenoic acid acid

In one preferred embodiment, the polyunsaturated fatty acid is docosatetraenoic acid.

Specifically, the present invention relates to a method for producing docosatetraenoic acid comprising

(a) isolating a nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95, 113 isolating another nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, 99 isolating a third nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 16-21, 101, 103 isolating a fourth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105, 107 and isolating a fifth nucleotide sequence having at least 75% identity to at least one of the nucleotide sequences set forth in SEQ ID NO: 19, 28, 29, 101;

(b) constructing at least one vector comprising said isolated nucleotide sequences of step (a);

(c) transforming the vector(s) of step (b) into a host cell for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a)

(d) exposing said host cell, to a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product, and

obtaining said docosatetraenoic acid acid

In one embodiment, the present invention relates to the use of specific nucleotide sequences encoding delta-12 desaturases, more specifically SEQ ID NOs 5 – 10, 93, 95, 113 which encode the amino acid sequences SEQ ID NOs 43-48. Usually these delta-12 desaturase-encoding nucleotide sequences are used together with at least 3 or more additional nucleotides sequences. The minimum three additional sequences are either

nucleotide sequences encoding delta-6 desaturase, delta-5 elongase, and delta-5 desaturase, or nucleotide sequences encoding delta-9 elongase, delta-8 desaturase and delta-5 desaturase. Additional sequences can be selected from sequences that are supplied in table 1. The same embodiment also relates to any delta-12 desaturase-encoding nucleotide sequence comprising or having at least 75% identity to any one of the nucleotide sequences SEQ ID NOs 5-10.

Specifically, a nucleotide sequence encoding a delta-12 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 5-10, 93, 95 and 113; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 5-10, 93, 95 and 113.

In the present context "delta-12 desaturase" relates to an enzyme that is capable of converting oleic acid to linoleic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to use of a specific nucleotide sequence encoding delta-9 elongase, more specifically SEQ ID NO 37, which encode the amino acid sequence SEQ ID NO 79. Usually this delta-9 elongase-encoding nucleotide sequence is used together with at least 3 or more additional nucleotide sequences. The minimum three additional sequences are nucleotide sequences encoding delta-12 desaturase, delta-8 desaturase and delta-5 desaturase. Additional sequences can be selected from sequences that are supplied in table 1. The same embodiment also relates to any delta-9 elongase-encoding nucleotide sequence that comprise or has at least 75% identity to the nucleotide sequence SEQ ID NO 37.

In the present context "delta-9 elongase" relates to an enzyme that is capable of converting linoleic acid to eicosadienoic acid and/or alpha linoleic acid to eicosatrienoic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to the use of specific nucleotide sequences encoding delta-9 desaturases, more specifically SEQ ID NOs 1-4, which encode the amino acid sequences SEQ ID NOs 39-42. Usually these delta-9 desaturase-

encoding nucleotide sequences are used together with at least 4 or more additional nucleotide sequences. The minimum four additional sequences are either nucleotide sequences encoding delta-12 desaturase, delta-9 desaturase, delta-8 desaturase and delta-5 desaturase, or nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase. Additional sequences can be selected from sequences that are supplied in table 1. The same embodiment also relates to any delta-9 desaturase-encoding nucleotide sequence comprising or having at least 75% identity to any one of the nucleotide sequences SEQ ID NOs 1-4.

Specifically, a nucleotide sequence encoding a delta-9 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 1-4; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NOs 1-4.

In the present context "delta-9 desaturase" relates to an enzyme that is capable of converting stearic acid to oleic acid and/or palmitic acid to palmitoleic acid, and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to the use of a specific nucleotide sequence encoding a delta-8 desaturases, more specifically SEQ ID NO 38, which endcodes the amino acid sequence SEQ ID NO 79. Usually delta-8 desaturase-encoding nucleotide sequences are used together with at least 3 or more additional nucleotides sequences. The minimum three additional sequences are nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, and delta-5 desaturase. Additional sequences can be selected from sequences that are supplied in table 1. The same embodiment also relates to any delta-8 desaturase nucleotide sequence comprising or having at least 75% identity to the nucleotide sequence SEQ ID NO 38.

In the present context "delta-8 desaturase" relates to an enzyme capable of converting eicosadienoic acid to dihomo-gamma linolenic acid or eicosatrienoic acid to eicosatrienoic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to use of specific nucleotide sequences encoding delta-6 desaturases, more specifically SEQ ID NOs 11-15, 97 and 99, which encode the amino acid sequences SEQ ID NOs 49-53, 98 and 100. Usually these delta-6 desaturase-encoding nucleotide sequences are used together with at least 3 or more additional nucleotide sequences. The minimum three additional sequences are nucleotide sequences encoding delta-12 desaturase, delta-6 elongase, and delta-5 desaturase. Additional sequences can be selected from sequences supplied in table 1. The same embodiment also relates to delta-6 desaturase-encoding nucleotide sequences comprising or having at least 75% identity to the nucleotide sequences SEQ ID NOs 11-15, 97 and 99.

Specifically, a nucleotide sequence encoding a delta-6 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 11-15, 97 and 99; and

b) nucleotide sequences having at least 75% identity to any one of the nucleotide sequences set forth in SEQ ID NOs 11-15, 97 and 99.

In the present context "delta-6 desaturase" is an enzyme capable of converting linoleic acid to gamma-linolenic acid and/or alpha-linolenic to stearidonic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to use of specific nucleotide sequences encoding delta-6 elongases, more specifically SEQ ID NOs 16-21, 101 and 103, which encode the amino acid sequences SEQ ID NOs 54-59, 102 and 104. Usually these delta-6 elongase-encoding nucleotide sequences are used together with at least 3 or more additional nucleotides sequences. The minimum three additional sequences are nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, and delta-5 desaturase. Additional sequences can be selected from sequences that are supplied in table 1. The same embodiment also relates to delta-6 elongase-encoding nucleotide sequences that comprise or have at least 75% identity to the nucleotide sequences SEQ ID NOs 16-21, 101 and 103.

Specifically, a nucleotide sequence encoding a delta-6 elongase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 16-21, 101 and 103; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NOs 16-21, 101 and 103.

In the present context "delta-6 elongase" relates to an enzyme capable of converting gamma-linoleic acid to dihomo-gamma-linolenic acid and/or stearidonic acid to eicosatetraenoic acid, and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to use of specific nucleotide sequences encoding delta-5 desaturases, more specifically SEQ ID NOs 22-27, 99, 105 and 107, which encode the amino acid sequences SEQ ID NOs 60-65, 100, 106 and 108. Usually these delta-5 desaturase-encoding nucleotide sequences are used together with at least 3 or more additional nucleotide sequences. The minimum three additional sequences are nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, and delta-6 elongase. Additional sequences can be selected from sequences that are supplied in table 1. The same embodiment also relates to delta-5 desaturase-encoding nucleotide sequences that comprise or have at least 75% identity to the nucleotide sequences SEQ ID NOs 22-27, 99, 105 and 107.

Specifically, a nucleotide sequence encoding a delta-5 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105 and 107; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105 and 107.

In the present context "delta-5 desaturase" relates to an enzyme capable of converting dihomo-gamma-linolenic acid to arachidonic and/or eicosatetraenoic acid to eicosapentaenoic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to use of specific nucleotide sequences encoding delta-5 elongases, more specifically SEQ ID NOs 19, 28-29 and 101, which encode the amino acid sequences SEQ ID NOs 66-67 and 102. In addition, it relates to nucleotide sequences encoding the amino acid sequence SEQ ID NO 68. Usually these delta-5 elongase-encoding nucleotide sequences are used together with at least 4 or more additional nucleotides sequences. The minimum four additional sequences are either nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase, or nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase and delta-5 desaturase. Additional sequences can be selected from sequences supplied in table 1. The same embodiment also relates to delta-5 elongase-encoding nucleotide sequences that comprise or have at least 75% identity to the nucleotide sequences SEQ ID NOs 19, 28-29 and 101 and to nucleotide sequences that encode amino acid sequences having at least 75% identity to SEQ ID NO 68.

Specifically, a nucleotide sequence encoding a delta-5 elongase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 19, 28-29 and 101; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NOs 19, 28-29 and 101; and

c) nucleotide sequences encoding amino acid sequences that have at least 75% identity to SEQ ID NO 68.

In the present context "delta-5 elongase" relates to an enzyme capable of converting arachidonic acid to docosatetraenoic acid and/or eicosapentaenoic acid to docosapentaeonic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to the use of specific nucleotide sequences encoding delta-4 desaturases, more specifically SEQ ID NOs 35-36 and 109, which encode the amino acid sequences SEQ ID NOs 74-75 and 110. In addition, it relates to nucleotide sequences encoding the amino acid sequences SEQ ID NOs 76-77.

Usually these delta-4 desaturase-encoding nucleotide sequences are used together with at least 4 or more additional nucleotides sequences. The minimum four additional sequences are either nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase, or nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase and delta-5 desaturase. Additional sequences can be selected from sequences supplied in table 1. The same embodiment also relates to delta-4 desaturase nucleotide sequences that comprise or have at least 75% identity to the nucleotide sequences SEQ ID NOs 35-36 and 109 and to nucleotide sequences that encode amino acid sequences having at least 75% identity to SEQ ID NOs 76-77.

Specifically, a nucleotide sequence encoding a delta-4 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 35-36 and 109; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NOs 35-36 and 109; and

c) nucleotide sequences encoding amino acid sequences that have at least 75% identity to SEQ ID NOs 76-77.

In the present context "delta-4 desaturase" relates to an enzyme capable of converting docosapentaenoic acid to docosahexaenoic acid and the meaning shall not exclude other functionality of said enzyme.

In one embodiment, the present invention relates to the use of specific nucleotide sequences encoding omega-3 desaturases, more specifically SEQ ID NOs 30-34, 87, 89 and 111, which encode the amino acid sequences SEQ ID NO 69-73, 88, 90 and 112. Usually these omega-3 desaturase-encoding nucleotide sequences are used together with at least 4 or more additional nucleotides sequences. The minimum four additional sequences are either nucleotide sequences encoding delta-12 desaturases, delta-6 desaturases, delta-6 elongase and delta-5 desaturase, or nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase and delta-5 desaturase. Additional sequences can be selected from sequences supplied in table 1. The same embodiment also relates to omega-3 desaturase-encoding nucleotide sequences that

comprise or have at least 75% identity to the nucleotide sequences SEQ ID NOs 30-34, 87, 89 and 111.

Specifically, a nucleotide sequence encoding an omega-3 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 30-34, 87, 89 and 111; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NOs 30-34, 87, 89 and 111.

In the present context "omega-3 desaturase" relates to an enzyme capable of converting linoleic acid to alpha-linolenic acid, gamma-linolenic aicd to stearidonic acid, eicosadieonic acid to eicosatrienoic acid, dihomo-gamma-linolenic acid to eicosatetraenoic aicd, arachidonic acid to eicosapentaenoic acid, and docosatetraenoic acid to docosapentaenoic acid or subsets of theses capabilities, and the meaning shall not exclude other functionality of said enzyme.

As commonly defined (se e.g. Encyclopaedia of Life Sciences, Nature Publishing Group, 2000) "identity" is here defined as sequence identity between genes or proteins at the nucleotide or amino acid level, respectively. Thus, in the present context "sequence identity" is a measure of identity between proteins at the amino acid level and a measure of identity between nucleic acids at nucleotide level. The protein sequence identity may be determined by comparing the amino acid sequence in a given position in each sequence when the sequences are aligned. Similarly, the nucleic acid sequence identity may be determined by comparing the nucleotide sequence in a given position in each sequence when the sequences are aligned.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at

that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions (e.g., overlapping positions) x 100). In one embodiment the two sequences are the same length.

One may manually align the sequences and count the number of identical amino acids. Alternatively, alignment of two sequences for the determination of percent identity can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm that can be utilised for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilised as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules. When utilising the NBLAST, XBLAST, and Gapped BLAST programs, the default parameters of the respective programs can be used. See http://www.ncbi.nlm.nih.gov. Alternatively, sequence identity can be calculated after the sequences have been aligned e.g. by the program of Pearson W.R and D.J. Lipman (Proc Natl Acad Sci USA 85:2444-2448, 1998) in the EMBL database (www.ncbi.nlm.gov/cgi-bin/BLAST). Generally, the default settings with respect to e.g. "scoring matrix" and "gap penalty" can be used for alignment. In the context of the present invention, the BLASTN and PSI BLAST default settings can be advantageous
The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

Heterologous Genes

The technology described within the present invention relates to genetically engineered non-plant host cells and host cells that produces PUFAs from e.g. non-fatty acid

substrates, such as sugar sources or combined fermentation substrates as described above.

The genetically transformed cells particularly harbour a heterologous oxygen-requiring pathway from stearic acid to PUFAs by expression of the following heterologous enzymes delta-9 desaturase, delta-12 desaturase, delta-9 elongase, delta-8 desaturase omega-3 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, delta-5 elongase, delta-4 desaturase or subsets hereof.

Delta-9 desaturase catalyses the reaction from palmitic acid to palimitoleic acid as well as the reaction from stearic acid to oleic acid. Delta-12 desaturase catalyzes the reaction from oleic acid to linoleic acid, which initiates the omega-6 pathway, linoleic acid is converted to gamma-linolenic acid by the action of delta-6 desaturase. Gamma- linolenic acid is elongated by two methyl groups by delta-6 elongase to form dihomo-gamma-linolenic, which is desaturated by delta-5 desaturase to arachidonic acid.

Alternatively, arachidonic acid can be produced from linoleic acid via the action of delta -9 elongase, delta-8 desaturase and delta-5 desaturase. Linoleic acid is elongated by delta-9 elongase to form eicosadienoic acid, which is converted to dihomo-gamma-linolenic acid via the action of delta-8 desaturase, and finally arachidonic acid is formed from dihomo-gamma-linolenic acid through the action of delta-5 desaturase. For both alternatives, arachidonic acid can be elongated by delta-5 elongase to form Δ7, Δ10, Δ13, Δ16-docosatetraenoic acid.

Omega-3 desaturase converts linoleic acids to alpha-linoleic acid, the starting point of the omega-3 pathway. Omega-3 desaturases are often highly unspecific and can convert gamma-linoleic acid to stearidonic acid, eicosadienoic acid to eicosatrienoic acid, dihomo-gamma-linolenic acid to eicosatetraenoic acid, arachidonic acid to EPA and docosatetraenoic acid to docosapentaenoic acid. The omega-3 pathway uses the same enzymes as the omega-6 pathway plus a delta-4 desaturase. Delta-6 desaturase catalyzes the reaction from alpha-linoleic acid to stearidonic acid, which is further converted by delta-6 elongase to eicosatetraenoic aicd. Alternatively, eicosatetraenoic acid can be produced from alpha-linolenic acid via the action of delta-9 elongase and delta-8 desaturase. Delta-9 elongase catalyzes the reaction from alpha-linolenic acid to eicosatrienoic acid, and delta-8 desaturase catalyses the reaction from eicosatrienoic acids to eicososatetraenoic acid. Desaturation of eicosatetraenoic acid by delta-5

desaturase leads to EPA. EPA is converted via delta-5 elongase to docosapentaenoic acid, which itself is desaturated by delta-4 desaturase to form DHA.

The heterologous genes can be isolated from any living organism, including fungi, plants, animals, algae and marine protists, amoeba and bacteria, that harbours pathways to oleic acid, linoleic acid, alpha-linolenic, gamma-linoleic acids, dihomo-gamma-linolenic acid, arachidonic acid, stearidonic acid, eicosatetraenoic acid, EPA, 7,10,13,16,19-docosapentaenoic acid, docosatetraeonic acid or DHA.

A non-exhaustive list of organisms that have such pathways leading to fatty acids with one or more double bond are bacteria such as but not limited to, *Spirulina spp., Synechocystis*, etc. fungi such as *Mortiella alpina, Mucor rouxii, Mucor circinelloides, Aspergillus fumigatus,* etc., plants like *Petroselinum crispum, Arabidopsis thaliana, Brassica napus, Glycine max, Zea mays, Ricinus communis, Corylus avellana, Phaeodactylum tricornutum*, etc., are the animals such as *Caenorhabditis elegans, Homo sapiens, Mus musculus, Rattus norvegicus, Lepidoptera,* etc., and the like algae such as *Schizochytrium, Thraustochytrium sp., Phaeodactylum tricornutum, etc.,* and amoeba,such as *Dictyostelium discoideum, etc.*

The expression of delta-12 desaturase has been reported in a wide range of different organisms including but not limited to, *Mortierella alpina, Mucor rouxii, Mucor circinelloides, Aspergillus fumigatus, Helianthus annuus, Petroselinum crispum, Arabidopsis thaliana, Brassica napus, Glycine max, Zea mays, Ricinus communis, Corylus avellana, Phaeodactylum tricornutum, C.elegans, Calendula officinalis* and cotton , but are not limited to these examples (WO9411516, US6025172, US2003/0180802, US2003/0172398, US2003/0074694, US2003/066104, US6,372,965, US6,441,278, WO200185968-A2, WO200179499-A1, US6372965-B1, WO200114538-A).

Delta-6 desaturases can be found at least in the following organisms *Mortierella alpina, Mucor rouxii, Mucor circinelloides, Pythium irregulare, Borago officinalis, Ceratodon purpureus, Physcomitrella patens, Anemone leveillei, Phaeodactylum tricornutum, Tetrahymena, Caenorhabditis elegans, Primulaceae, Homo sapiens, Castor,* evening primrose, *Synechocystis, Spirulina spp., Physcomitrella patens* (WO 9927111, US 2002/0170090, WO 03/072784, US 6,492,108, US 6,686,186, US 2002/0151019, US 2002/0108147, WO 02/081702, WO200272028-A2, US6355861-B1, WO200144485-A, JP2001095588-A, WO200120001-A, WO200102591-A, WO200104636-A, JP2001095588-

A, WO200175069-A1). Delta-6 elongases have been identified among others in the following organisms *Mortierella alpina, Physcomitrella patens, Caenorhabditis elegans, Mortiella Alpina, Homo sapiens, C.elegans, Mus musculus, T.aureum, Pavlova, Thraustochytrium aureum, Phytophthora infestans* (US6403349, WO 03/102138, US 2003/0177508, WO200244320-A, WO200208401-A, WO200159128-A, DE10005973-A1, WO200055330-A, WO2003064638-A2).

Delta-9 elongases have been isolated from *Isochrysis galbana* (WO02077213, Qi et al. 2004) and delta-8 desaturases from *Euglena gracilis* (Wallis and Browse 1999). Delta-5 desaturases have been isolated from *Mortierella alpina, Phytophthora megasperma, Physcomitrella patens, Phaeodactylum tricornutum, Thraustochytrium sp. ATCC 2165, Caenorhabditis elegans, Dictyostelium discoideum, Schizochytrium, Thraustocytrium aureum, Saprolegnia diclina, Isochrysis galbana, Phytophthora megasperma, Homo sapiens*, rat, *Euglena* , among others (US5972664, WO9933958, WO9846765, WO 02/081668, WO2003012092-A, US6428990-B1, US6432684-B1, WO200234940-A, WO200040705-A, WO200034439-A, WO200104636-A, WO2003012092-A).

Omega-3 desaturases can be isolated from plants, fungi, and nematodes, such as *Petroselinum crispum, Brassica napus, Arabidopsis thaliana, Glycine soya, Saprolegnia diclina, Caenorhabditis elegans* (i.e. US6194167, US20030196217, Yadav et al. 1993, Kirsch et al. 1997) *or from Saccharomyces kluyveri*

Delta-5 elongase have been found among others in mouse, *Homo sapiens, Caenorhabditis elegans, Thraustochytrium aureum,*(i.e. US 2003/0177508 and WO200208401-A) and Delta-4 desaturase can be isolated from fungi, algae and marine protists including *Thraustochytrium sp., Euglena gracilis, Thraustochytrium aureum, Saprolegnia diclina, Isochrysis galbana*, etc. but are not limited to these organisms (i.e. WO 02/090493, WO200226946-A, Qiu et al. 2001, Meyer et al. 2003)

In one embodiment, the present invention describes the simultaneous heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase in a microorganism, which leads to the production of PUFAs, and in particular production of arachidonic acid from non-fatty acid substrates. The genes can be expressed on a single plasmid or several plasmids, such as one plasmid, two plasmids, three plasmids, four plasmids, five plasmids, six plasmids, seven plasmids, eight pladsmids, nine plasmids, or ten plasmids or more. The use of a single plasmid carrying

several heterologous genes, for example four genes, is advantageous because it ensures that the cells that carry the plasmid contain all heterologous genes. In contrast if several plasmids are used, a fraction of the cell population will contain only one of the plasmids and thus will not express the full heterologous pathway. However, the number of heterologous genes that can be expressed from a single plasmid is limited by the increased size of the plasmid; large plasmids tend to be less stable in the cell than small plasmids, which leads to poorer expression from large plasmids. A presently preferred embodiment therefore involves expression of one or two heterologous genes per plasmid.

Furthermore, it describes the simultaneous heterologous expression of genes encoding delta-12 desaturase, omega-3 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase in a microorganism, which results in production of EPA and other PUFAs from non-fatty acid substrates.

The invention also describes the simultaneous heterologous expression of genes encoding delta-12 desaturase, omega-3 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, delta-5 elongase and delta-4 desaturase in a microorganism, which results in production of DHA from non-fatty acid substrates.

The invention describes an additional expression of a delta-9 desaturase. The production of PUFAs in microorganism can be improved by expression of delta-9 desaturases that are more specific for the production of oleic acid rather than palmitoleic acid (Figure 2). Recently, two delta-9 desaturases from *M. alpina*, *ole1* and *ole2*, have been cloned. Both genes complement yeast Δ*ole1* mutants, which cannot grow without supplementation of 16:1 and 18:1 fatty acids in the medium. Both *M. alpina* delta-9 desaturases shift the fatty acid content from 16:1 desaturated fatty acid towards 18:1 desaturated fatty acid (oleic acid) in yeast. The oleic acid content of the Δ*ole1* yeast expressing *M. alpina ole1* was 53.6% of total lipid, compared to 21.6% in wild-type *S. cerevisiae* (Wongwathanarat et al., 1999). The present invention shows that expression of a heterologous delta-9 desaturase together with the heterologous PUFA biosynthetic pathway can increase production of PUFA in yeast. For example, the expression of *Mortierella alpina ole1* together with a *Mortierella alpina* delta-12 desaturase in yeast increases the production of linoleic acid, for example by the factor of 5 or any multiples thereof.

Nucleotide sequences and constructs

By "gene" is meant a nucleotide sequence, also referred to as DNA or RNA sequence, which encodes a specific protein. Nucleotide sequences encoding described PUFA desaturases and elongases can be isolated from their natural sources using standard procedures known in the art. One such procedure comprises isolation of total RNA, reverse transcription using Oligo(dT) or random primers followed by PCR amplification using sequence-specific primers. Novel PUFA desaturase- and elongase encoding nucleotide sequences can likewise be isolated by known procedures. Preferentially, these are based on sequence homology and comprise, for example, PCR using degenerate primers and screening of DNA or cDNA libraries by colony hybridization using radiolabeled polynucleotide probes. Alternatively, the isolation methods are based on the function of the polypeptide encoded by the polynucleotide. For example, a cDNA expression library is generated from a PUFA-producing organism and screened for desaturation or elongation of PUFA substrates.

PCR (Polymerase Chain Reaction) is a technique for the synthesis of large quantities of specific DNA sequences that is based on repeated cycles of in vitro replication of DNA template by a temperature-tolerant DNA polymerase (Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51: 263-273 (1986); European Patent Application 50424; European Patent Application 84796; European Patent Application 258017: European Patent Application 237362; European Patent Application 201184; US4683202; US4582788; US4683194). The technique utilizes sets of specific in vitro synthesized oligonucleotides, termed primers, which anneal to complementary sequences on the template DNA and prime DNA synthesis by DNA polymerase. Amplification is achieved by applying several cycles (normally 20-50) of melting of the double-stranded template at high temperature, annealing of the primers, and DNA replication. For amplification of known sequences, primers are usually designed to match the template sequence exactly. However, desired features, such as specific restriction sites, can be introduced into the resulting DNA fragment through the design of the primers. Moreover, a specific 5' tail sequence can be included in the primer sequence, which later allows fusion of the PCR-product to a DNA fragment containing a matching 3´end sequence.

PCR using degenerate primers can be used to amplify a novel DNA sequence with sequence homology to known DNA sequences. The primers are then designed to match DNA regions of high homology, as deduced from multiple alignments of known sequences. The primers are allowed to contain different bases at certain positions, such that the primer used in the PCR reaction is actually a mix of oligonucleotides with different sequences. A portion of the oligonucleotides in the mix anneal to the target sequence, allowing amplification of the template.

Techniques for manipulation of nucleic acids encoding PUFA enzymes such as subcloning nucleic acid sequences encoding polypeptides into expression vectors, labeling probes, DNA hybridization, and the like are described generally in Sambrook et al., Molecular cloning: A laboratory manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989).

Following isolation of a desired gene, it can be sequenced using known methods.

Once a desired nucleotide sequence has been isolated, it can be expressed in a host cell. For the purpose of expressing a heterologous nucleic acid in a host cell, it is operably linked to a promoter and a terminator sequence using standard cloning techniques or standard in vitro procedures, such as fusion by PCR.

The term "promoter" refers to a DNA sequence capable of controlling the expression of a gene. The promoter sequence consists of proximal and more distal elements located upstream of the gene. The more distal elements are often referred to as enhancers. Promoter sequences can also be located within the transcribed portion of the DNA sequence, and/or downstream of the transcribed sequences. The "terminator sequence", also called the 3´non-coding sequence refer to DNA sequences located downstream of a gene and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The promoter and terminator sequences used for heterologous expression can be derived from the native sequence of the heterologous gene. More often, the promoter and terminator sequences are taken from a highly expressed DNA sequence of the host cell. For example, suitable promoter sequences for expression in *Saccharomyces cerevisiae* include the constitutive promoters of *TDH3, ADH1, TPI1, ACT1 GPD* and *PGI* or the promoter of any constitutively and highly transcribed yeast gene and the galactose-inducible promoters of *GAL1, GAL10* and *GAL7*. Also contemplated by the present invention are other yeast inducible promoters, such as but not limited to, the *CUP1* metallothionein promoter, which enables gene expression in the presence of heavy metals, such as copper (Karin M, et al, (1984) Proc Natl Acad Sci USA 81(2): 337-41. The *MET15* promoter can also be used when repression of genes is desired. Suitable bacterial promoter sequences are for example the promoter and operator region of the *E. coli* tryptophan biosynthetic pathway as described by Yanofsky (1984) *J. Bacteriol.*, 158:1018-1024 and the promoter of phage lambda (Pλ) as described by Herskowitz and Hagen, (1980) *Ann. Rev. Genet.*, 14:399-445. A large number of terminator sequences are known and have been found to be satisfactory in a variety of hosts from the same and different genera and species. A heterologous polynucleotide, operably linked to a promoter and a terminator sequence, is hereafter termed an expression cassette.

The term operably linked refers to the association of a gene with a sequence that controls its expression on a single nucleic acid fragment. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence.

Constructs containing heterologous genes of interest can be introduced into the host by standard techniques. These techniques include transformation, such as for example, in *S. cerevisiae*, lithium acetate transformation, spheroplasting, and use of a *kar1Δ15* mutant (Georgieva, B. et al, (2002) Meth. Enzymol. 350: 278-89),, protoplast fusion, lipofection, transfection, transduction, conjugation, infection, bolistic impact, electroporation, or any other method that introduces the foreign DNA into the host cell. For simplicity, a host cell manipulated in this way will be referred to as "transformed", "recombinant" or "genetically modified". The construct that is introduced into the host cell contains in addition to the expression cassette a marker gene, which allows identification of transformed cells and, in the case of extrachromosomal expression, also prevents the cell from losing the construct. Preferably, the marker gene encodes a conditionally essential gene, which has been deleted in the host genome. Examples of the latter are, in yeast, the *URA3* gene, the *TRP1* gene, the *HIS3* gene and the *LEU2* gene, which restore the ability of the *ura3*, *trp1*, *his3* or *leu2* mutant yeast cell to produce the essential compounds uracil, tryptophane, histidine and leucine, respectively. Recombinant yeast cells can therefore be selected and maintained on medium lacking these factors. Alternatively, the marker gene can confer resistance to an antibiotic, allowing selection and maintenance of recombinant cells in medium containing the antibiotic.

Table 1. Examples of desaturases and elongases, useful for heterologous PUFA production.

Enzyme	Source	Reference	SEQ ID NO: (nucleotide)	SEQ ID NO: (amino acid)
delta-9 desaturase	*Mortierella alpina*	present patent application	1	39
	Cryptococcus curvatus	Meesters et al. 1996 *Yeast* 12:723-730	2	40
	Histoplasma capsulatus	Gargano et al. 1995 *Lipids* 30:899-906	3	41
	Trichoplusia ni	Liu et al. 1999 *Insect Biochem Mol Biol* 29: 435-443	4	42
delta-12 desaturase	*Mortierella alpina*	present patent application	5	43
	Mucor rouxii	Passorn et al. 1999 *Biochem Biophys Res Commun* 263: 47-51	6	44
	Mucor circinelloides	BAB69056, GenBank Dec 2000	7	45

	Aspergillus fumigatus	CAE47978, GenBank Dec 2003	8	46
	Cryptococcus curvatus	AAS78627, Pubmed March 2004	9	47
	Caenorhabditis elegans	US 2003/0172398	10	48
	Aspergillus parasiticus	Wilson et al. 2004 *Microbiology* 150: 2881-2888	93	94
	Pichia pastoris	AAX20125, Pubmed March 2005	95	96
	Saccharomyces kluyveri	Watanabe et al. 2004 *Biosci Biotechnol Biochem* 68: 721-727	113	114
delta-6 desaturase	*Mortierella alpina*	present patent application	11	49
	Mucor rouxii	Laoteng et al. 2000 *Biochem Biophys Res Commun* 279: 17-22	12	50
	Borago officinalis	Sayanova et al. 1997 *Proc Natl Acad Sci USA* 94: 4211-4216.	13	51
	Anemone leveillei	Whitney et al. 2003 *Planta* 217:983-92	14	52
	Caenorhabditis elegans	WO 9927111	15	53
	Marchantia polymorpha	Kajikawa et al. 2004 *Plant Mol Biol* 54: 335-352	97	98
	Cyprinus carpio	Hastings et al. 2001 *Proc Natl Acad Sci* 98: 14304-14309	99	100
delta-6 elongase	*Mortierella alpina*	present patent application	16	54
	Physcomitrella patens	WO0159128	17	55
	Caenorhabditis elegans	WO200055330-A	18	56
	Mus musculus	WO200208401-A	19	57
	Thraustochytrium aureum	WO200208401-A	20	58
	Phytophthora infestans	WO2003064638-A2	21	59
	Salmo salar	Hastings et al. 2004 *Mar Biotechnol* 6: 463-474	101	102
	Marchantia polymorpha	Kajikawa et al. 2004 *Plant Mol Biol* 54: 335-352	103	104
delta-5	*Mortierella alpina*	present patent application	22	60

desaturase	*Phytohphtora megasperma*	WO03012092	23	61
	Thraustochytrium	WO200226946-A	24	62
	Caenorhabditis elegans	WO9933958	25	63
	Pythium irregulare	WO200226946-A	26	64
	Phaedodactylum. tricornutum	US20040053379-A1	27	65
	Salmo salar	Hastings et al. 2004 *Mar Biotechnol* 6: 463-474	105	106
	Marchantia polymorpha	Kajikawa et al. 2004 *Plant Mol Biol* 54: 335-352	107	108
	Cyprinus carpio	Hastings et al. 2001 *Proc Natl Acad Sci* 98: 14304-14309	99	100
delta-5 elongase	*Mus musculus*	Tvrdik et al. 2000 J Cell Biol 149: 707-717	28	66
	Mus musculus	WO200208401-A	19	57
	Homo sapiens	WO0244320	29	67
	Pavlova	WO 03102138		68
	Salmo salar	Hastings et al. 2004 *Mar Biotechnol* 6: 463-474	101	102
omega-3 desaturase	*Caenorhabditis elegans*	US6194167	30	69
	Petroselinum crispum	Kirsch et al. 1997 *Proc Natl Acad Sci USA* 94: 2079-2084	31	70
	Arabidopsis thaliana	Yadav et al. 1993 *Plant Physiol* 103: 467-476	32	71
	Brassica napus	Yadav et al. 1993 *Plant Physiol* 103: 467-476	33	72
	Glycine soya	Yadav et al. 1993 *Plant Physiol* 103: 467-476	34	73
	Mortierella alpina	Sakuradani et al. 2005 *Appl Microbiol Biotechnol* 66: 648-654	89	90
	Saccharomyces kluyveri	Oura et al. 2004 *Microbiology* 150: 1983-1990	87	88
	Saprolegnia diclina	Pereira et al. 2004 *Biochem J* 378: 665-671	111	112
delta-4 desaturase	*Thraustochytrium aureum*	WO200226946-A	35	74
	Euglena gracilis	Meyer et al. 2003 *Biochemistry* 42: 9779-9788	36	75
	Isochrysis galbana	WO 02/090493		76

	Schizochytrium aggregatum	WO 02/090493		77
	Pavlova lutheri	Pereira et al. 2004 *Biochem. J.* 384: 357-366	109	110
delta-9 elongase	*Isochrysis galbana*	WO2002077213_A2	37	78
delta-8 desaturase	*Euglena gracilis*	WO200034439-A	38	79

In one embodiment of the invention, the genes required for PUFA production are integrated into the genome of the host organism. Integration of heterologous polynucleotide sequences into the genome of *Saccharomyces cerevisiae* by homologous recombination is a well known, standard technique for genetic manipulation of *S. cerevisiae*. A linear DNA construct can be targeted for integration at any location in the yeast genome by fusing it to target sequences at the 5′ end and at the 3′ end. Upon transformation with the linear DNA construct, the DNA-double strand break repair pathway of yeast is activated, mediating homologous recombination between the target sequences of the linear DNA substrate and the corresponding sequences in the yeast genome.

This results in integration of the linear DNA construct into the genome, and simultaneous looping out of any sequence between the two target sequences in the yeast genome. Depending on the purpose of the genetic manipulation, target sequences can be selected on each side of a yeast gene, resulting in knock-out of that gene, or adjacent to each other, resulting in disruption of the target sequence but otherwise leaving the genome intact.

The present invention involves the integration of several heterologous genes, encoding PUFA desaturases and elongases. Preferably, all expression cassettes necessary for the production of a specific PUFA are assembled on a single plasmid, which also contain a marker gene and a target sequence for integration. The target sequence is engineered to contain or naturally contains a unique restriction site, which allows linearization of the plasmid.

Following transformation of yeast with the linearized plasmid, the yeast cells are plated on selection medium as described herein and recombinant cells containing the heterologous DNA construct are identified.

Preferably, all expression cassettes necessary for the production of a specific PUFA are assembled on a single construct and are simultaneously integrated into the genome of the yeast cell. However, if expression of many heterologous genes is desired, it may be beneficial to place the individual genes on several, for example two, different constructs, targeted for

integration at different sites in the host genome as described above. Following identification of the recombinant cells, the two separate chromosomal integrations can be combined by crossing of the recombinant strains. If necessary, each recombinant strain is first taken through an intermediate cross in order to introduce suitable genetic markers.

Crossing of strains is a traditional, widely used and very efficient method for combining different genotypes. In short, two haploid yeast strains of opposite mating type (i.e., for *S. cerevisiae*, mating types are denoted as *MAT*a and *MAT*alpha) are allowed to mate on rich medium, such as YPD. Usually, the haploid strains each display a different selectable phenotype, such as amino acid auxotrophy, allowing for diploids to be selected on double drop-out medium. Alternatively, the cells can be plated on rich medium following mating, and diploids are identified by inducing cells to undergo meiosis and sporulation by simply transferring a number of single colonies onto sporulation medium, such as, for example, medium containing potassium acetate as the sole carbon source,and monitoring sporulation by microscopy. Following sporulation of the diploids, the spores are dissected and the genotypes of the resulting haploid strains are scored using various methods, such as replica plating to suitable drop-out plates and by colony-PCR. Crossing of strains to combine different genotypes can also be advantageously accomplished by using a mutant that is defective for karyogamy, such as the *kar1Δ15* mutant (Georgieva, B. et al (2002) Meth. Enzymol. 350: 278-89).

Preferably, different promoters are used in the construction of the several expression cassettes in the heterologous construct, so as to avoid further homologous recombination events to take place and loop out parts of the heterologous construct. Alternatively, a promoter sequence is placed in two copies on the same heterologous construct but in divergent directions so that a direct repeat is avoided.

Well-known methods for improving heterologous expression include codon-optimization of the heterologous nucleotide sequence. This is done by employing the host-preferred codons, as determined from codons of the highest frequency in highly expressed proteins of the host of interest. The coding sequence for a polypeptide having PUFA desaturase or elongase activity can be chemically synthesized in whole or in part using methods well established in the literature. Moreover, the nucleotide sequence surrounding the translational start-codon ATG has been found to influence gene expression in yeast. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequence of the heterologous gene can be modified to include an efficient yeast translation initiation sequence to obtain optimal gene expression. This can be accomplished by standard techniques such as PCR-based site directed mutagenesis or by fusion to the initiation sequence of a highly expressed yeast gene.

Production of PUFA-containing bakers yeast

Recombinant yeast strains containing heterologous pathways to PUFAs, such as arachidonic acid or DHA, can be grown in batch, fed batch or chemostat cultivation as described in example 10 in order to produce high amounts of PUFA-containing biomass. Following harvest of biomass, e.g. by centrifugation or filtration, and possible drying of the biomass to a suitable degree, it can be used as a functional food ingredient, for example as bakers yeast, yeast extract or as a flavour enhancer. The PUFA-containing biomass can also be used directly as a functional food, for example in tablets as an alternative to fish oil capsels.

Thus, the present invention also relates to food products, such as funtional food products, wherein said food product has an increased content of polyunsaturated fatty acids when compared to a product produced by a cell, that is not modified for heterologous expression according to the present invention.

Strategies for improved PUFA yields in recombinant yeast

The yield of PUFA in a recombinant yeast can be improved via several strategies, some of which involve increasing the PUFA percentage of total fatty acid, and others that involve metabolic engineering of the host for increased fatty acid production.

Increasing the percentage of PUFA in total fatty acid

One strategy that can be used for increasing the percentage PUFA in total fatty acid involves heterologous expression of a delta-9 desaturase with substrate specificity for stearic acid rather than palmitic acid. Expression of such a delta-9 desaturase shifts the fatty acid composition towards higher concentration of oleic acid, the precursor of the PUFA pathway, and results in increased PUFA production (see examples 9 and 12). Another strategy that can increase oleic acid content in yeast involves overexpression of the genes *ELO1*, *ELO2* and/or *ELO3*, which encode fatty acid elongases. Overexpression of these genes may increase the concentration of fatty acids with 18 carbon atoms in relation to the concentration of fatty acids with 16 carbon atoms. Alternatively, a heterologous elongase with substrate specificity for palmitic acid or palmitoleic acid can be expressed in order to increase the availability of fatty acids with 18 carbon atoms.

Furthermore, the efficiency of expression of heterologous genes encoding enzymes in the PUFA pathway can be increased by optimizing the codons of the heterologous genes. Heterologous genes are likely to contain codons that are rare in the host organism, and the availability of the corresponding tRNAs may therefore be limiting for expression of the gene in question. In order to optimize the codons of a specific gene, the corresponding amino acid sequence is back-translated using the optimal codon frequency of the host. This can be done, for example, using the Backtranslation tool V2.0 program. The coding and non-coding strand of the codon-optimized gene can then be chemically synthesized in the form of overlapping oligonucleotides. To assemble the synthetic gene, the overlapping oligonucleotides are allowed to hybridize to each other and reconstitute the full, double-stranded nucleotide sequence, which can then be amplified by PCR.

Metabolic engineering for increased fatty acid production

In *S. cerevisiae*, fatty acid synthesis is carried out by the fatty acid synthase (FAS) complex (Figure 3), which consists of a heteromultimeric complex of two multifunctional subunits (α and β). Overexpression of the α and β subunits, encoded by the yeast genes *FAS2* and *FAS1*, respectively, can substantially increase the fatty acid content of *S.cerevisiae* and thereby the yield of PUFA on cell dry-weight

Acetyl-CoA carboxylase catalyses the reaction from acetyl-CoA to malonyl-CoA and is encoded by the *ACC1* gene product. Overexpression of *ACC1* allows an increase in the malonyl-CoA pool, and thereby effecting more efficient fatty acid synthesis. Consequently, the lipid and PUFA yield in *ACC1* overexpression mutants is increased (example 39).

Other targets for metabolic engineering include genes involved in the synthesis of the storage lipid triacylglycerol (TAG). In yeast, TAG synthesis is achieved by the action of enzymes encoded by the four genes *DGA1*, *LRO1*, *ARE1* and *ARE2* (Sandager et al. 2002 J Biol Chem 277: 6478-6482). Overexpression of these genes can therefore increase the content of TAG, and thereby total fatty acid content, of yeast. In particular, overexpression of *DGA1*, encoding an acyl-CoA:diacylglycerol acyltransferase, can increase the TAG content, as deletion of this gene alone results in approximately 60% decrease in the TAG content and approximately 40% decrease in the total lipid content (Sandager et al. 2002 J Biol Chem 277: 6478-6482) and example 29.

Furthermore, the cellular content of TAG and total lipid can be increased by increasing the availability of precursors needed for TAG synthesis. For example, the intracellular concentration of the main TAG precursor L-glycerol 3-phosphate can be increased more than 20 times in yeast by overexpressing *GPD1*, encoding glycerol 3-phosphate dehydrogenase, and deleting *GPP1* and *GPP2*, which encode isoenzymes of glycerol 3-phosphatase (Nguyen et al. 2004 Met Eng 6: 155-163). Potentially the same strategy can be used overexpressing GPD2 or GPD1 and GPD2 together with a deletion of GPP1 and GPP2.

In order to produce more TAG, other target genes can be overexpressed such as genes involved in phosphatidic acid production. Here, synthesis of lysophosphatidic acid and phosphatidic acid can be increased by overexpression of *GAT1* and *SLC1* encoding L-glycerol 3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase, respectively. The phosphatidic acid pool is increased and more precursor is available to increase the levels of TAG (example 30). Other target genes include *SPO14*, a phospholipase D that catalyses the reaction of phosphatidylcholine to phosphatidic acid and choline (Xie, et al, (1988) Proc. Natl. Acad. Sci USA 95(21):12346-51.

The availability of the main precursor for fatty acid synthesis, acetyl-CoA, can be increased by expressing a heterologous ATP:citrate lyase. ATP:citrate lyase is present in most oleaginous organisms but usually not in non-oleaginous yeast such as *Saccharomyces cerevisiae* and catalyzes the conversion of citrate into acetyl-CoA and oxaloacetate (Figure 5). Furthermore, heterologous expression of an AMP-regulated isocitrate dehydrogenase is likely to lead to accumulation of citrate during conditions of nitrogen limitation (Ratledge 2002 Biochem Soc Trans 30:1047-1050). The combined expression of heterologous genes encoding ATP:citrate lyase and an isocitrate dehydrogenase whose activity is favoured by the presence of AMP can therefore lead to increased availability of acetyl-CoA and increased fatty acid production in the host cell.

It has been shown that overexpression of panthothenate kinase in *Escherichia coli* gives rise to higher CoA levels. Hence, overexpression of the putative panthothenate kinase in *Saccharomyces cerevisiae* encoded by *YDR531W*may allow an increase in the CoA pool in *Saccharomyces cerevisiae* and thereyby increase production of acetyl-CoA.

Fatty acid synthesis by the FAS complex requires NADPH as a cofactor, and increased fatty acid production may therefore result in a redox imbalance in the cell, such that the availability of NADPH controls the rate of fatty acid production. Several strategies can be used to overcome this problem, including expression of a heterologous non-phosphorylating $NADP^+$ dependent glyceraldehyde 3-phosphate dehydrogenase (Bro et al. 2005 under review) and modification of the ammonium assimilation pathway (Nissen et al. 2002 Met Eng 2: 69-77). Overexpression of *FAS1* and *FAS2* can be combined with deletion of *GDH1* and overexpression of either *GDH2*, encoding an NADH-dependent glutamate dehydrogenase, or *GLT1* and *GLN1*, which encode the enzymes that constitute the GS-GOGAT pathway of ammonium assimilation (Figure 6)

The fatty acid yield can furthermore be increased by deleting the structural gene for fatty acid degradation, in yeast *POX1* (Figure 7). Such a deletion can be combined, for example, with the overexpression or integration of a heterologous steaoryl-CoA desaturase that is more specific in synthezing oleic acid instead of palmitoleic acid, thus favoring the synthesis of PUFAs (figure 3) This can further be combined with overexpression of ACC1 to further enhance the production of PUFA and/or lipids.

Another possibility to improve the PUFA production or yield is to enhance the elongation PUFA. For example, the delta-6 elongase in the pathway to ARA performs a condensation reaction, where 18:3 is fused to a malonyl-CoA unit. However, the complete elongation of 18:3 to 20:3 additionally involves a keto group reduction, a dehydration and an enoyl reduction (Figure 8). Since heterologous expression of delta-6 elongase in yeast results in elongation of 18:3, the three latter reactions must be catalyzed by endogenous yeast enzymes. The function of these enzymes in wild type yeast is the formation of very long chain fatty acids and the elongation of exogenous short chain fatty acids. A β-ketoacyl-CoA reductase is encoded by the gene YBR159W. The gene encoding the β-hydroxyacyl-CoA dehydrase has not been identified in yeast. The final step of fatty acid elongation, enoyl reduction, is catalyzed by the enzyme encoded by the TSC13 gene. To increase the elongation efficiency, YBR159W and TSC13 can therefore be overexpressed.

Lipid yield and/or PUFA content can also be improved further by combining the differents metabolic engineering strategies described above. This can be done by crossing of strains carrying different genetic modifications, for example as described in Examples 28, 35 and 36.

Overexpression of native yeast genes described above can be achieved by replacing the native promoter with a strong constitutive promoter, for example the TDH3 promoter, the ADH1 promoter, the *ACT1* promoter, the TPI promoter or the GPD promoter, using an approach similar to the strategy used in the present invention for integration of *M. alpine ole1* (example 7). Likewise, heterologous genes can be expressed through integration into the genome as described in example 7. Alternatively, native and heterologous genes can be expressed from plasmids such as the yeast episomal plasmids 2μ and CEN plasmids, or yeast integrating plasmids (i.e., YiP series), as described in examples 2-5. Deletion of yeast genes can be achieved by an approach similar to the one described in example 7. The different genetic alterations described in the present example can be combined by crossing of the recombinant strains or by combining chromosomal modifications with expression from vectors, which can result in an efficient engineered host for production of PUFAs.

The skilled addressee will recognize that simple expression of a heterologous PUFA pathway in bakers yeast is expected to result in a low content of arachidonic acid, as bakers yeast has a low content (approximately 10% of cell dry weight) of fatty acids. Furthermore, the fatty acids in bakers yeast primarily consists of fatty acids with 16 carbon atoms, and the most dominant mono-unsaturated fatty acid is palmitoleic acid, which can not serve as a precursor for synthesis of arachidonic acid. The result of simply expressing four genes encoding the enzymes of the pathway to arachidonic acid in *S. cerevisiae* is illustrated herein (Example 12) where expression of these four genes results in an arachidonic acid content of 0.8% of the fatty acids, or corresponding to less than 0.08% of the yeast dry weight.

Thus, the present invention relates to improvement of the PUFA content in the host organism through fermentation optimization (i.e. fermentation using nitrogen limitation, phosphor limitation, trace element limitation, NaCl limitation, myo-inositol limitation, etc.), e.g. decreasing the temperature and/or designing an optimal medium, or through improving the flux towards fatty acids by metabolic engineering, e.g. through overexpression of fatty acid synthases, over-expression of other enzymes involved in biosynthesis of the precursors for PUFAs, or codon optimization of the heterologous genes, or expression of heterologous enzymes involved in the biosynthesis of the precursor for PUFAs, i.e. oleic acid.

Thus, a preferred embodiment of the present invention relates to a method according to the present invention, wherein said host cell, such as *Saccharomyces cerevisiae* is cultivated in a myo-inositol deficient medium.

Growth on myo-inositol deficient medium

It is known that in some yeast species grown on media deficient in myo-inositol, the lipid yield is increase. Hence, it is of advantage to grow the genetically modified cells of this invention on a medium that is not supplemented with myo-inositol such that the lipid and PUFA yield is increased.

Codon usage and optimization

Codon usage can often differ among different species. For the expression of a heterologous protein from an organism that has a different codon usage it is of advantage to alter the codon usage of the heterologous protein to match that of the host cell. Thereby protein expression can be improved. For example, as compared to *Saccharomyces cerevisiae,* the codon usage is different in many fungi, such as *Mortierella alpina, Cryptococcus, curvatus, and Histoplasma capsulatus, Mucor rouxii, Mucor circinelloides, Aspergillus fumigatus Saccharomyces klyveri, Phytophthera megasperma, Pythium irregulareand Aspergillus parasiticus, insects, such as Trichoplusia ni, mammals, such as Mus musculus and Homo sapiens, algae, such Thraustocytrium aureum, Euglena gracilis, Isochrysis galbana, Saprilegnia diclian, Phaeodactylum tricornutum, Saprolegnia and Schizochytrium aggregatum and Pavlova lutheri, worms, such as Caenorhabditis elegans, plants, such as Arabidopsis thaliana, Brassica napus, Glycine soya, Borago officinalis, Anemone leveillei, Marchantia polymorpha, Physcomitrella patens, Petroselinum crispum and Phytophthora infestans, fish, such as Cyprinus carpio,* and *Salmo salar.* Hence, codon optimization of nucleotide sequences of the corresponding enzymes mentioned in Table 1 will increase PUFA production.

Thus, a preferred embodiment of the present invention relates to a method according to the present invention, wherein said heterologous nucleotide sequences are codon optimized for expression in *Saccharomyces cerevisiae*.

Furthermore, in one embodiment the present invention relates to a method according to the present invention, wherein said combined heterologous expression further comprises

an over-expression of at least one of the genes selected from the group consisting of ACC1, YBR159W, ELO1, ELO2, ELO3, FAS1, FAS2, DGA1, LRO1, ARE1, ARE2, and GPD1.

Another embodiment relates to a method according to the present invention, wherein said combined heterologous expression further comprises a deletion of at least one of the genes selected from the group consisting of GPP1, GPP2 and POX1.

Another embodiment relates to a method according to the present invention, wherein said combined heterologous expression further comprises a heterologous expression of the nucleotide sequences encoding ATP:citrate lyase and/or an isocitrate dehydrogenase which is stimulated by AMP.

Another embodiment relates to a method according to the present invention, wherein said combined heterologous expression further comprises a heterologous expression of a nucleotide sequence encoding a non-phosphorylating NADP-dependent D-glyceraldehyde-3-phosphate dehydrogenase.

Another embodiment relates to a method according to the present invention, wherein said combined heterologous expression further comprises a deletion of the gene GDH1 and optionally an over-expression of at least one of the genes selected from the group consisting of GDH2, GLN1 and GLT1.

Another embodiment relates to a method according to the present invention, wherein said combined heterologous expression further comprises an over-expression of at least one of the genes selected from the group consisting of TSC13, GAT1, SLC1 and YDR531W.

Thus, in one embodiment, the present invention relates to methods, cells, and compositions relating to an improved polyunsaturated fatty acid content, wherein said heterologous expression increases the content of each individual specific polyunsaturated fatty acid, particularly ARA, EPA and DHA, to more than 2 % of the total fatty acid content, such as 3% of the total fatty acid content, 4% of the total fatty acid content, 5% of the total fatty acid content, 6% of the total fatty acid content, 7% of the total fatty acid content, 8% of the total fatty acid content, 9% of the total fatty acid content, 10% of the total fatty acid content or more.

Thus, in one presently particular preferred embodiment, the method of the invention discloses heterologous expression which increases the content of arachidonic acid, eicosapentaenoic acid and/or docosahexaenoic acid to more than 2 % of the total fatty acid content in the genetically modified *Saccharomyces cerevisiae* described herein.

In another embodiment the present invention relates methods, cells, and compositions relating to an improved polyunsaturated fatty acid content, wherein said heterologous expression increases the content of each individual specific polyunsaturated fatty acid to more than 0.1% of the yeast dry weight, such as 0.2% of the yeast dry weight, 0.3% of the yeast dry weight, 0.4% of the yeast dry weight, 0.5% of the yeast dry weight, 0.6% of the yeast dry weight, 0.7% of the yeast dry weight, 0.8% of the yeast dry weight, 0.9% of the yeast dry weight, 1% of the yeast dry weight, 2% of the yeast dry weight, 3 of the yeast dry weight, 4% of the yeast dry weight, 5% of the yeast dry weight or more.

Vector

The polynucleotides encoding PUFA desaturases and elongases can be expressed in the host organism from extrachromosomal elements. For extrachromosomal expression in e.g. yeast, high copy number plasmids, are preferred. Other yeast vectors include yeast replicating plasmids (YRps), such as the 2μ plasmid, which have a chromosomally derived replicating sequence and are propagated in medium copy-number (20 to 40 copies per cell), and yeast centromere plasmids (Ycps; also known as CEN plasmids),, which have both a replication origin and a centromere sequence, ensuring stable segregation. Several yeast expression vectors with differing selection markers can be used in combination when the purpose is to express several heterologous genes. In addition, several heterologous genes can be expressed from the same plasmid, for example using the pESC vectors (Stratagene), which permit simultaneous, inducible expression from the divergent GAL1/GAL10 promoter sequence. A variety of prokaryotic expression systems can be used to express PUFA-synthesizing desaturases and elongases, including the pBR322 plasmid, the pUC plasmids and derivatives thereof. For expression in prokaryotes the heterologous genes are assembled in an artificial operon, meaning that a single promoter sequence controls the expression of a cluster of genes. Several genes encoding PUFA-synthesizing desaturases and elongases can be fused by PCR and subsequently be subcloned into a bacterial expression vector using standard techniques.

Thus, one aspect of the present invention relates to a vector comprising at least 4 isolated nucleotide sequences having at least 75% sequence identity to the nucleotide sequences selected from the group consisting of SEQ ID NO: 1-38.

As described in detail above, the combined expression of 4-7 heterologous nucleotide sequences should enable PUFA production. Thus, the vector can comprise nucleotide sequences encoding, for example, delta-12 desaturase, delta-6 desaturase, delta 6 elongase and delta-5 desaturase or, for example, delta-12 desaturase,delta-9 elongase, delta-8 desaturase and delta-5 desaturase. Alternatively the expression vector can, for example, comprise 7 genes encoding delta-12 desaturase, delta-6 desaturase, delta 6 elongase, delta-5 desaturase, omega-3 desaturase, delta-5 elongase and delta-4 desaturase.

Due to cross-feeding between cells, it is generally not expected that all cells in a population contain a specific plasmid construct, even though selection pressure is being applied. This effect is enhanced if several different vectors with different selection markers are used. Therefore, all genes required for PUFA production are preferably expressed from a single expression vector. However, as large vector constructs (i.e. vectors exceeding approximately 20 kb in size) may be unstably replicated and segregated in the host cell, it can also be beneficial to express the heterologous pathway from several, for example two, separate vectors.

As the skilled addressee would recognise, the individual nucleotide sequences can be expressed either from a single vector or from separate vectors. The skilled artisan is also well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression, and thus that multiple events must be screened in order to obtain strains displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis, such as analysis of fatty acid compositionthat can be detected by methods such as but not limited to, high-performance liquid chromatography (HPLC), gas chromatography coupled to mass spectrometry (GC-MS), thin-layer chromatography, among others.

Preferably, the heterologous genes are expressed from several vectors. It can also be advantageous to express one or several heterologous genes in the PUFA pathway from a genomic location. For example, eicosapentaenoic acid can be produced in *S. cerevisiae* by expressing five heterologous genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase and omega-3 desaturase from in total three different vectors, and additionally expressing a heterologous delta-9 desaturase from a genomic location (Example 58).

As noted above, once the vector has been constructed, it may then be introduced into the host cell of choice by methods known to those of ordinary skill in the art including, for example, transfection, transformation and electroporation.

The host cell is then cultured under suitable conditions permitting expression of the genes leading to the production of the desired PUFA, which is then recovered and purified.

The genetically modified cell

In another aspect, the present invention relates to a genetically modified cell comprising a vector according to the present invention.

As indicated, a further embodiment of the present invention relates to a genetically modified cell, wherein expression of said isolated nucleotide sequences from said vector results in said cell producing a polyunsaturated fatty acid that is not produced in a wild-type of said host cell.

Composition

In one aspect the present invention relates to a composition comprising a polyunsaturated fatty acid produced by a genetically modified cell according to the present invention.

In a preferred embodiment the composition comprising a polyunsaturated fatty acid produced by a genetically modified *Saccharomyces cerevisiae* according to the present invention.

As exemplified below, compositions containing 25% PUFA in total fatty acid composition through heterologous expression of at least 4 genes can be achieved by the methods of the present invention.

Thus, in a presently preferred embodiment, the invention relates to a composition comprising at least 25% polyunsaturated fatty acid produced in total fatty acid composition by a genetically modified cell according to the invention.

However, even smaller amounts are of both economical and technical importance, thus the invention furthermore relates to a composition comprising at least 2 % polyunsaturated fatty acid of the total fatty acid composition, such as 5% polyunsaturated fatty acid acid of the total fatty acid composition, such as 10% or more polyunsaturated fatty acid acid of the total fatty acid composition.

Indeed higher levels are even more preferred such as 25% polyunsaturated fatty acid of the total fatty acid composition, such as 30% polyunsaturated fatty acid of the total fatty acid composition, such as 35% polyunsaturated fatty acid of the total fatty acid composition, such as 40% polyunsaturated fatty acid of the total fatty acid composition, such as 45% polyunsaturated fatty acid of the total fatty acid composition, such as 50% polyunsaturated fatty acid of the total fatty acid composition, such as 55% polyunsaturated fatty acid of the total fatty acid composition, such as 60% polyunsaturated fatty acid of the total fatty acid composition, such as 65% polyunsaturated fatty acid of the total fatty acid composition, such as 70% polyunsaturated fatty acid of the total fatty acid composition, such as 75% polyunsaturated fatty acid of the total fatty acid composition, such as 80% polyunsaturated fatty acid of the total fatty acid composition, such as 85% polyunsaturated fatty acid of the total fatty acid composition, such as 90% polyunsaturated fatty acid of the total fatty acid composition, such as 95% polyunsaturated fatty acid of the total fatty acid composition, such as 97% polyunsaturated fatty acid v total fatty acid composition, such as 98% polyunsaturated fatty acid of the total fatty acid composition, such as 99% polyunsaturated fatty acid of the total fatty acid composition, such as 100% polyunsaturated fatty acid v total fatty acid composition, produced from a cell, such as a microorganism that expresses a heterologous pathway leading to mono unsaturated fatty acids and particularly PUFAs.

A composition in the context of the present invention shall mean a blend or mixture of compounds.

In one embodiment said composition is an oil.

As described above the PUFA can be of various formations/formulations, thus in one embodiment said polyunsaturated fatty acid is in a formation of triacylglycerides.

In another embodiment, said polyunsaturated fatty acids are in a formulation of phospholipids.

In a further embodiment said polyunsaturated fatty acids is in a formulation of free fatty acids.

Use of composition of the present invention

There is now numerous data on the advantages of PUFA. Clinical evidence has been collected that shows that DHA and ARA are advantageous in the development of neural and retinal functions and could therefore be of benefit to babies to achieve improved memory and eyesight. In addition, preterm and young infants are actually unable to synthesize sufficient amounts of DHA and naturally receive PUFAs by breast milk. However, PUFAs have previously been absent in infant formula as well as in cow milk. DHA also reduces or eliminates the risk factor involved in various diseases like cardiovascular diseases and has some positive effects on hypertension, arthritis, arteriosclerosis and thrombosis. It is now established that both PUFAs are increasingly supplied in food, for example in infant formula, and also in pharmaceutical and cosmetics formulations. This increasing demand can be covered with the technology described within the present invention through the supply of PUFA such as an oil comprising triacylglycerides, phospholipids or free fatty acids (enriched in PUFA) that are produced in reproducibly high and constant quality by a genetically modified *Saccharomyces cerevisiae* that is capable of producing PUFAs with four or more double bonds when grown on a non-fatty acid substrate.

A general source of PUFAs is fish oil. However, the fatty acid content of fish oil varies during the fishing season and in some cases the fish oil may be contaminated because of environmental pollution. Besides this, fish oil has an obnoxious smell. Fish itself does not produce PUFA but takes it up usually through the consumption of algae. Nowadays, fish

oil rich in PUFAs is produced from aquacultured fish. However, the PUFA content can vary depending on the diet that they are fed. Besides this, a shortage in high quality fish feed is expected and it is therefore of advantange to supplement fish feed with PUFA or simply with yeast or feed that is high in PUFA content.

Thus, one embodiment of the present invention relates to the use of a composition according to the present invention as an ingredient in a food product.

Another embodiment relates to the use of a composition according to the present invention as an ingredient in a cosmetic product.

In a particular preferred embodiment, the present invention relates to the use of a composition according to the invention as an ingredient in feed.

In a presently most preferred embodiment, the present invention relates to the use of a genetically modified *Saccharomyces cerevisiae* according to the invention as an ingredient in feed.

General remarks

The composition could be an oil containing polyunsaturated fatty acids, and the PUFAs can be in formation of triglycerides, phospholipids or free fatty acids.

BRIEF DESCRIPTION OF THE DRAWINGS

The following Detailed Description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, wherein:

Figure 1
Synthesis of polyunsaturated fatty acids in a genetically engineered microorganism (omega-6 delta/delta-6 and omega-3 delta/delta-6 pathway using delta-6 desaturase and delta-6 elongase)

Figure 2
Synthesis of polyunsaturated fatty acids in a genetically engineered microorganism (omega-6 delta/delta-8 and omega-3 delta/delta-8 pathway using delta-9 elongase and delta-8 desaturase)

Figure 3
Simplified view of fatty acid biosynthesis in *S. cerevisiae*

Figure 4
Pathway to TAG and phospholipids in *Saccharomyces cerevisiae*. PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol.

Figure 5
Pathway to cytosolic acetyl-CoA in oleagineous yeast and fungi.

Figure 6
Ammonia assimilation in *Saccharomyces cerevisiae*

Figure 7
Fatty acid degradation by beta-oxidation

Figure 8
Fatty acid elongation

Figure 9
A: Yeast vector for expression of genes encoding delta-12 desaturase and delta-6 desaturase.

B: Yeast vector for expression of genes encoding delta-6 elongase and delta-5 desaturase.

Figure 10
Strategy for integration of *M. alpina ole1* into the genome of S. *cerevisiae*

Figure 11
Gas chromatogram profile of fatty acids, extracted from *S. cerevisiae* harbouring a heterologous pathway including delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase and corresponding mass spectrogram of arachidonic acid

Figure 12
Gas chromatogram profile of fatty acids, extracted from *S. cerevisiae* harbouring a heterologous pathway including delta-9 desaturase, delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase and corresponding mass spectrogram of arachidonic acid

Figure 13
A: Plasmid map over pWAD1
B: Plasmid map over pWAD2

Figure 14
A: Plasmid map over pWJ716-TD1
B: Plasmid map over pWJ716-TD2

Figure 15
Method used for overexpression of genes by promoter replacement. YFG, arbitrary gene to be overexpressed.

Figure 16
Strategy for integration of *M. alpina ole1* into the genome of *S. cerevisiae* at the *POX1* locus

Figure 17
Strategy for integration of *Sordaria macrospora acl1* and *acl2* into the genome of *Saccharomyces cerevisiae*.

Figure 18
Overview of the performance of genetically modified strains of *Saccharomyces cerevisiae*: yield of arachidonic acid on carbon source plotted against percentage arachidonic acid of total fatty acid.

Figure 19
Construction of plasmid p300

Figure 20
A: Plasmid map over pESC-LEU-SK33
B: Plasmid map over pESC-LEU Ssc2
C: Plasmid map over pESC-LEU-Ssc2-SK33

Figure 21
Gas chromatogram profile of fatty acids, extracted from *S. cerevisiae* strain FS01446, harbouring a heterologous pathway including delta-9 desaturase, delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase and omega-3 desaturase and corresponding mass spectrogram of eicosapentaenoic acid.

Figure 22
Method used for assembly of synthetic gene encoding delta-4 desaturase, codon optimized for expression in *Saccharomyces cerevisiae*.

Figure 23
Plasmid map over pESC-LEU-Ssc2-delta-4d

Figure 24
Strategy for integration of *Saccharomyces kluyveri FAD3*, encoding an omega-3 desaturase, into the genome of *Saccharomyces cerevisiae*.

SEQUENCE ORIGIN

SEQ ID NO: 1 is a nucleotide sequence of from *Mortierella alpina* encoding a delta- 9 desaturase
SEQ ID NO: 2 is a nucleotide sequence of from *Cryptococcus curvatus* encoding a delta-9 desaturase
SEQ ID NO: 3 is a nucleotide sequence of from *Histoplasma capsulatus* encoding a delta-9 desaturase
SEQ ID NO: 4 is a nucleotide sequence of from *Trichoplusia ni* encoding a delta-9 desaturase

SEQ ID NO: 5 is a nucleotide sequence of from *Mortierella alpina* encoding a delta- 12 desaturase

SEQ ID NO: 6 is a nucleotide sequence of from *Mucor rouxii* encoding a delta- 12 desaturase

SEQ ID NO: 7 is a nucleotide sequence of from *Mucor circinelloides* encoding a delta- 12 desaturase

SEQ ID NO: 8 is a nucleotide sequence of from *Aspergillus fumigatus* encoding a delta- 12 desaturase

SEQ ID NO: 9 is a nucleotide sequence of from *Cryptococcus curvatus* encoding a delta- 12 desaturase

SEQ ID NO: 10 is a nucleotide sequence of from *Caenorhabditis elegans* encoding a delta- 12 desaturase

SEQ ID NO: 11 is a nucleotide sequence of from *Mortierella alpina* encoding a delta- 6 desaturase

SEQ ID NO: 12 is a nucleotide sequence of from *Mucor rouxii* encoding a delta- 6 desaturase

SEQ ID NO: 13 is a nucleotide sequence of from *Borago officinalis* encoding a delta- 6 desaturase

SEQ ID NO: 14 is a nucleotide sequence of from *Anemone levellei* encoding a delta- 6 desaturase

SEQ ID NO: 15 is a nucleotide sequence of from *Caenorhabditis elegans* encoding a delta- 6 desaturase

SEQ ID NO: 16 is a nucleotide sequence of from *Mortierella alpina* encoding a delta- 6 elongase

SEQ ID NO: 17 is a nucleotide sequence of from *Physcomitrella patens* encoding a delta- 6 elongase

SEQ ID NO: 18 is a nucleotide sequence of from *Caenorhabditis elegans* encoding a delta- 6 elongase

SEQ ID NO: 19 is a nucleotide sequence of from mouse encoding a delta- 6 elongase

SEQ ID NO: 20 is a nucleotide sequence of from *Thraustochytrium aureum* encoding a delta- 6 elongase

SEQ ID NO: 21 is a nucleotide sequence of from *Phytophthora infestans* encoding a delta- 6 elongase

SEQ ID NO: 22 is a nucleotide sequence of from *Mortierella alpina* encoding a delta- 5 desaturase

SEQ ID NO: 23 is a nucleotide sequence of from *Phytophthora megasperma* encoding a delta- 5 desaturase

SEQ ID NO: 24 is a nucleotide sequence of from *Thraustochytrium* sp. ATCC 21685 encoding a delta- 5 desaturase

SEQ ID NO: 25 is a nucleotide sequence of from *Caenorhabditis elegans* encoding a delta- 5 desaturase

SEQ ID NO: 26 is a nucleotide sequence of from *Pythium irregulare* encoding a delta- 5 desaturase

SEQ ID NO: 27 is a nucleotide sequence of from *Phaeodactylum tricornutum* encoding a delta-5 desaturase

SEQ ID NO: 28 is a nucleotide sequence of from mouse encoding a delta- 5 elongase

SEQ ID NO: 29 is a nucleotide sequence of from human encoding a delta- 5 elongase

SEQ ID NO: 30 is a nucleotide sequence of from *Caenorhabditis elegans* encoding an omega-3 desaturase

SEQ ID NO: 31 is a nucleotide sequence of from *Petroselinum crispum* encoding an omega-3 desaturase

SEQ ID NO: 32 is a nucleotide sequence of from *Arabidopsis thaliana* encoding an omega-3 desaturase

SEQ ID NO: 33 is a nucleotide sequence of from *Brassica napus* encoding an omega-3 desaturase

SEQ ID NO: 34 is a nucleotide sequence of from *Glycine soya* encoding an omega-3 desaturase

SEQ ID NO: 35 is a nucleotide sequence of from *Thraustochytrium aureum* encoding a delta-4 desaturase

SEQ ID NO: 36 is a nucleotide sequence of from *Euglena gracilis* encoding a delta-4 desaturase

SEQ ID NO: 37 is a nucleotide sequence of from *Isochrysis galbana* encoding a delta-9 elongase

SEQ ID NO: 38 is a nucleotide sequence of from *Euglena gracilis* encoding a delta-8 desaturase

SEQ ID NO: 39 is the amino acid sequence encoded by SEQ ID NO:1

SEQ ID NO: 40 is the amino acid sequence encoded by SEQ ID NO:2

SEQ ID NO: 41 is the amino acid sequence encoded by SEQ ID NO:3

SEQ ID NO: 42 is the amino acid sequence encoded by SEQ ID NO:4

SEQ ID NO: 43 is the amino acid sequence encoded by SEQ ID NO:5

SEQ ID NO: 44 is the amino acid sequence encoded by SEQ ID NO:6

SEQ ID NO: 45 is the amino acid sequence encoded by SEQ ID NO:7

SEQ ID NO: 46 is the amino acid sequence encoded by SEQ ID NO:8

SEQ ID NO: 47 is the amino acid sequence encoded by SEQ ID NO:9

SEQ ID NO: 48 is the amino acid sequence encoded by SEQ ID NO:10

SEQ ID NO: 49 is the amino acid sequence encoded by SEQ ID NO:11

SEQ ID NO: 50 is the amino acid sequence encoded by SEQ ID NO:12

SEQ ID NO: 51 is the amino acid sequence encoded by SEQ ID NO:13
SEQ ID NO: 52 is the amino acid sequence encoded by SEQ ID NO:14
SEQ ID NO: 53 is the amino acid sequence encoded by SEQ ID NO:15
SEQ ID NO: 54 is the amino acid sequence encoded by SEQ ID NO:16
SEQ ID NO: 55 is the amino acid sequence encoded by SEQ ID NO:17
SEQ ID NO: 56 is the amino acid sequence encoded by SEQ ID NO:18
SEQ ID NO: 57 is the amino acid sequence encoded by SEQ ID NO:19
SEQ ID NO: 58 is the amino acid sequence encoded by SEQ ID NO:20
SEQ ID NO: 59 is the amino acid sequence encoded by SEQ ID NO:21
SEQ ID NO: 60 is the amino acid sequence encoded by SEQ ID NO:22
SEQ ID NO: 61 is the amino acid sequence encoded by SEQ ID NO:23
SEQ ID NO: 62 is the amino acid sequence encoded by SEQ ID NO:24
SEQ ID NO: 63 is the amino acid sequence encoded by SEQ ID NO:25
SEQ ID NO: 64 is the amino acid sequence encoded by SEQ ID NO:26
SEQ ID NO: 65 is the amino acid sequence encoded by SEQ ID NO:27
SEQ ID NO: 66 is the amino acid sequence encoded by SEQ ID NO:28
SEQ ID NO: 67 is the amino acid sequence encoded by SEQ ID NO:29
SEQ ID NO: 68 is the amino acid sequence of a delta-5 elongase from *Pavlova*
SEQ ID NO: 69 is the amino acid sequence encoded by SEQ ID NO:30
SEQ ID NO: 70 is the amino acid sequence encoded by SEQ ID NO:31
SEQ ID NO: 71 is the amino acid sequence encoded by SEQ ID NO:32
SEQ ID NO: 72 is the amino acid sequence encoded by SEQ ID NO:33
SEQ ID NO: 73 is the amino acid sequence encoded by SEQ ID NO:34
SEQ ID NO: 74 is the amino acid sequence encoded by SEQ ID NO:35
SEQ ID NO: 75 is the amino acid sequence encoded by SEQ ID NO:36
SEQ ID NO: 76 is the amino acid sequence of a delta-4 desaturase from *Isochrysis galbana*
SEQ ID NO: 77 is the amino acid sequence of a delta-4 desaturase from *Schizochytrium aggregatum*
SEQ ID NO: 78 is the amino acid sequence encoded by SEQ ID NO:37
SEQ ID NO: 79 is the amino acid sequence encoded by SEQ ID NO:38
SEQ ID NO: 80 is a nucleotide sequence from *Sordaria macrospora* encoding subunit 1 of ATP:citrate lyase
SEQ ID NO: 81 is the amino acid sequence encoded by SEQ ID NO: 80
SEQ ID NO: 82 is a nucleotide sequence from *Sordaria macrospora* encoding subunit 2 of ATP:citrate lyase
SEQ ID NO: 83 is the amino acid sequence encoded by SEQ ID NO: 82
SEQ ID NO: 84 is a synthetic nucleotide sequence encoding a delta-4 desaturase, codon-optimized for expression in *S. cerevisiae*

SEQ ID NO: 85 is a synthetic nucleotide sequence encoding a delta-9 elongase, codon-optimized for expression in *S. cerevisiae*

SEQ ID NO: 86 is a synthetic nucleotide sequence encoding a delta-8 desaturase, codon-optimized for expression in *S. cerevisiae*

SEQ ID NO: 87 is a nucleotide sequence from *Saccharomyces kluyveri* encoding an omega-3 desaturase

SEQ ID NO: 88 is the amino acid sequence encoded by SEQ ID NO: 87

SEQ ID NO: 89 is a nucleotide sequence from *Mortierella alpina* encoding an omega-3 desaturase

SEQ ID NO: 90 is the amino acid sequence encoded by SEQ ID NO: 89

SEQ ID NO: 92 is the amino acid sequence encoded by SEQ ID NO: 91

SEQ ID NO: 93 is a nucleotide sequence from *Aspergillus parasiticus* encoding a delta-12 desaturase

SEQ ID NO: 94 is the amino acid sequence encoded by SEQ ID NO: 93

SEQ ID NO: 95 is a nucleotide sequence from *Pichia pastoris* encoding a delta-12 desaturase

SEQ ID NO: 96 is the amino acid sequence encoded by SEQ ID NO: 95

SEQ ID NO: 97 is a nucleotide sequence from *Marchantia polymorpha* encoding a delta-6 desaturase

SEQ ID NO: 98 is the amino acid sequence encoded by SEQ ID NO: 97

SEQ ID NO: 99 is a nucleotide sequence from *Cyprinus carpio* encoding a delta-6/delta-5 desaturase

SEQ ID NO: 100 is the amino acid sequence encoded by SEQ ID NO: 99

SEQ ID NO: 101 is a nucleotide sequence from *Salmo salar* encoding a delta-6/delta-5 elongase

SEQ ID NO: 102 is the amino acid sequence encoded by SEQ ID NO: 101

SEQ ID NO: 103 is a nucleotide sequence from *Marchantia polymorpha* encoding a delta-6 elongase

SEQ ID NO: 104 is the amino acid sequence encoded by SEQ ID NO: 103

SEQ ID NO: 105 is a nucleotide sequence from *Salmo salar* encoding a delta-5 desaturase

SEQ ID NO: 106 is the amino acid sequence encoded by SEQ ID NO: 105

SEQ ID NO: 107 is a nucleotide sequence from *Marchantia polymorpha* encoding a delta-5 desaturase

SEQ ID NO: 108 is the amino acid sequence encoded by SEQ ID NO: 107

SEQ ID NO: 109 is a nucleotide sequence from *Pavlova lutheri* encoding a delta-4 desaturase
SEQ ID NO: 110 is the amino acid sequence encoded by SEQ ID NO: 109
SEQ ID NO: 111 is a nucleotide sequence from *Saproleigna diclina* encoding a omega-3 desaturase
SEQ ID NO: 112 is the amino acid sequence encoded by SEQ ID NO: 111
SEQ ID NO: 113 is a nucleotide sequence from *Saccharomyces kluyveri* encoding a delta-12 desaturase
SEQ ID NO: 114 is the amino acid sequence encoded by SEQ ID NO: 113

EXAMPLES

Example 1

Isolation of genes encoding delta-9 desaturase, delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase

The fungus Mortierella alpina produces arachidonic acid via a pathway, where oleic acid is desaturated and elongated in turn by a delta-12 desaturase, a delta-6 desaturase, a delta-6 elongase and a delta-5 desaturase. The nucleotide sequences encoding these enzymes were amplified by PCR using first strand cDNA from *Mortierella alpina* CBS 608.70. In addition, the nucleotide sequence coding for the delta-9 desaturase of M. alpina was isolated. The defined primers used for the amplification were designed to match the published sequences of *M. alpina* genes encoding these enzymes.

The procedure was as follows:
M. alpina CBS 608.70 was cultivated in 100 ml GY medium (20 g/L glucose, 10 g/L yeast extract pH 6.0) at room temperature for 3 days. Biomass was collected by filtration and total RNA was isolated using Trizol reagent (Gibco BRL). Approximately 5 µg of RNA was used for reverse transcription (Superscript II RT, Invitrogen) using Oligo(dT)12-18 as primer. After first strand cDNA sythesis, complementary RNA was removed by RNAse digestion. The cDNA was then used as template for PCR (Phusion enzyme, Finnzymes) using the following primers: 5´ATGGCAACTCCTCTTCCCCCCTCC 3´ and 5´CTATTCGGCCTTGACGTGGTCAGTGC 3´ for delta-9 desaturase;

5′AACCCTTTTTCAGGATGGCACC 3′ and 5′AAAGTTGTGTCCGGTAAATGCTTC 3′ for delta-12 desaturase; 3′ GGACTAGTCCACCATGGCTGCTGCTCCCAGTGTGAGG 5′ and 3′ CCATCGATGGCTTACTGTGCCTTGCCCATCTTGGAGG 5′
for delta-6 desaturase; 5′ATGGAGTCGATTGCGCCATTCC 3′ and 5′TTACTGCAACTTCCTTGCCTTCTCC3′ for delta-6 elongase; and 5′ATGGGTACGGACCAAGGAAAAACC3′ and 5′CTACTCTTCCTTGGGACGGAGTCC3′ for delta-5 desaturase. The resulting fragments of the expected sizes were excised from an agarose gel and purified using GFX-columns (Amersham).

Example 2

Construction of a yeast vector for expression of delta-12 desaturase

The gene encoding delta-12 desaturase, isolated as described in Example 1, was reamplified by PCR using the primers 5′GACCTCGAGTAAGCTTATGGCACCTCCCAACACTATTG 3′ and 5′GCTAGCCGCGGTACCAATTACTTCTTGAAAAAGACC 3′. These primers introduced XhoI and NheI restriction sites at the 5′ and 3′ ends of the gene, respectively, and allowed ligation of the XhoI/NheI restricted PCR fragment into an XhoI/NheI digested pESC-TRP vector (Stratagene) to yield pESC-TRP-delta-12. The sequence of the gene encoding delta-12 desaturase (SEQ ID NO 5) was obtained by sequencing of two different clones of pESC-TRP-delta-12.

Example 3

Construction of a yeast vector for expression of delta-12 desaturase and delta-6 desaturase

The gene encoding delta-6 desaturase was isolated as described in Example 1. The resulting PCR fragment contained SpeI and ClaI restriction sites at the 5′ and 3′ ends of the gene, respectively. The fragment was restricted with SpeI and ClaI and was ligated into SpeI/ClaI digested pESC-TRP-delta-12 (Example 2). The resulting plasmid, pESC-TRP-delta-12 delta-6, contained the genes encoding delta-12 desaturase and delta-6 desaturase under the control of the divergent GAL1/GAL10 promoter (figure 9A). The sequence of the gene encoding delta-6 desaturase (SEQ ID NO 11) was obtained by sequencing of two different clones.

Example 4

Construction of a yeast vector for expression of delta-6 elongase

The gene encoding delta-6 elongase, isolated as described in Example 1, was reamplified by PCR using the primers 5´ GACCTCGAGTAAGCTTATGGAGTCGATTGCGCC 3´and 5´ GCTAGCCGCGGTACCAATTACTGCAACTTCCTTGC 3´. These primers introduced HindIII and NheI restriction sites at the 5´ and 3´ ends of the gene, respectively, and allowed ligation of the HindIII/NheI restricted PCR fragment into a HindIII/NheI digested pESC-URA vector (Stratagene) to yield pESC-URA-elo. Two different clones of pESC-URA-elo were sequenced to obtain the sequence of the cloned gene (SEQ ID NO 16).

Example 5

Construction of a yeast vector for expression of delta-6 elongase and delta-5 desaturase

The gene encoding delta-5 desaturase, isolated as described in Example 1, was reamplified by PCR using the primers 5´CGCACTAGTATCGATATGGGTACGGACCAAGG 3´ and 5´ TTAATTAAGAGCTCAGATCTTCTACTCTTCCTTGGGACG 3´. These primers introduced ClaI and SacI restriction sites at the 5´ and 3´ ends of the gene, respectively, and allowed ligation of the ClaI/SacI restricted PCR product into ClaI/SacI digested pESC-URA-elo (Example 4). The resulting plasmid, pESC-URA-elo-delta-5, contained the genes encoding delta-6 elongase and delta-5 desaturase under the control of the divergent GAL1/GAL10 promoter (figure 9B). The sequence of the gene encoding delta-5 desaturase (SEQ ID NO 22) was obtained by sequencing of two different clones of pESC-URA-elo-delta-5.

Example 6

Expression of the pathway to arachidonic acid in yeast

Yeast strains containing the appropriate genetic markers were transformed with the vectors described in Examples 2, 3, 4, and 5, separately or in combination. Transformants were selected on medium lacking uracil and tryptophane and subsequently streak purified on the same medium.

S. cerevisiae strain CEN.PK113-3C (MATa trp1) was transformed separately with the vector pESC-TRP-delta-12 (Example 2), yielding the strain FS01321, and with pESC-TRP-delta-12 delta-6 (Example 3), resulting in the strain FS01322. *S. cerevisiae* strain FS01267 (*MATa trp1 ura3*) was co-transformed with pESC-TRP-delta-12 delta-6 and pESC-URA-elo (Example 4), and the transformed strain was named FS01323. The same strain was also co-transformed with pESC-TRP-delta-12 delta-6 and pESC-URA-elo-delta-5 (Example 5), resulting in the strain FS01324.

Example 7

Replacement of yeast OLE1 with M. alpina ole1

Replacement of the native *S. cerevisiae* OLE1 gene with the corresponding gene from M. alpina was carried out through homologous recombination with a bipartite substrate (Figure 10). One part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the TDH3 promoter sequence, the *M. alpina* ole1 gene and a target sequence downstream of the native *S. cerevisiae* OLE1. The second part of the bipartite substrate consisted of a target sequence upstream of the native OLE1, fused to the TDH3 promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which the native OLE1 had been knocked out and replaced with two copies of the TDH3 promoter sequence as a direct repeat on either side of the *K. lactis URA3* marker gene and the M. alpina ole1 gene immediately downstream of the second TDH3 promoter repeat. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the *URA3* gene. This resulted in a strain, in which the native *S. cerevisiae* OLE1 gene had been replaced with the *M. alpina* ole1 under the control of theTDH3 promoter. Suitable genetic markers were introduced into this strain by crossing it with a strain of opposite mating type and containing the desired marker, inducing sporulation, dissecting the spores, and scoring the genotypes of the novel haploid strains.

The procedure was as follows:

For construction of the first part of the bipartite gene targeting substrate, the *M. alpina* ole1 gene (SEQ ID NO 1), isolated as described in Example 1, was reamplified by PCR

using the primers 5´ATGGCAACTCCTCTTCCCCCCTCC 3´ and 5´TTGTTATTGTAATGTGATACCTATTCGGCCTTGACGTGG 3´. A target sequence downstream of *S. cerevisiae* OLE1 was amplified by PCR using *S. cerevisiae* genomic DNA as template and the primers 5´ GTATCACATTACAATAACAAAACTGCAAC 3´ and 5´ ACCAGCATCTATTAAAGTAAAATACCG 3´. A third DNA fragment was generated by PCR using a plasmid, containing the TDH3 promoter sequence (-1 to -1067) downstream of the K. lactis *URA3*, as template and the primers 5´ CTTGACGTTCGTTCGACTGATGAGC 3´ and 5´ GGGGGGAAGAGGAGTTGCCATTTTGTTTGTTTATGTGTG 3´. These PCR fragments were then fused during two rounds of PCR. First, *M. alpina* ole1 was fused to the downstream target sequence using the primers 5´ATGGCAACTCCTCTTCCCCCCTCC 3´ and 5´ ACCAGCATCTATTAAAGTAAAATACCG 3´. Second, the product of the first fusion reaction was fused to the *K. lactis URA3*/TDH3 promoter fragment using the primers 5´ CTTGACGTTCGTTCGACTGATGAGC 3´ and 5´ ACCAGCATCTATTAAAGTAAAATACCG 3´. This resulted in the fusion product 2/3URA3-TDH3p-ole1-DOWN, which constituted the first part of the bipartite gene targeting substrate.

For construction of the second part of the bipartite substrate, a target sequence upstream of the native *S. cerevisiae* OLE1 was amplified by PCR using *S. cerevisiae* genomic DNA as template and the primers 5´ GCTGAAAAGATGATGTTCTGAGG 3´ and 5´ AGTACATACAGGGAACGTCCGCGGTCTGCAGAGAAGGC 3´. A second PCR fragment was constructed using a plasmid, containing the TDH3 promoter sequence (-1 to -1067) upstream of the *K. lactis URA3*gene, as template and the primers 5´ GGACGTTCCCTGTATGTACTAAAAATGAAAGAAGCTTACCAG 3' and 5´ GAGCAATGAACCCAATAACGAAATC 3´. Finally, this fragment was fused to the upstream target sequence by PCR using the primers 5´ GCTGAAAAGATGATGTTCTGAGG 3´ and 5´ GAGCAATGAACCCAATAACGAAATC 3´, resulting in the fusion product UP-TDH3p-2/3URA3, which was the second part of the bipartite gene targeting substrate.

The yeast strain CEN.PK 113-5D (MATa ura3) was transformed with the linear substrates 2/3URA3-TDH3p-ole1-DOWN and UP-TDH3p-2/3URA3 and plated out on medium lacking uracil. Transformants were streak purified on the same medium and then transferred onto medium containing 5-FOA. Pop-out recombinants were streak purified on 5-FOA-containing medium and verified by colony PCR. The correct integration and absence of mutations in the TDH3 promoter and *M. alpina* ole1 (SEQ ID NO 1) was verified by sequencing of the modified region in two different transformants. The resulting strain, FS01309, had the genotype MATa OLE1::TDH3p-*M.alpina* ole1.

To introduce the *TRP1* marker marker into FS01309, FS01309 was crossed with a trp1 strain of the same genetic background (CEN.PK) and opposite mating type. Diploids were selected on medium lacking uracil and tryptophane and transferred to sporulation medium. Following sporulation, spores were dissected using a Singer MSM microscope and micromanipulator dissection microscope. Tetrads were scored for auxotrophy by replica-plating to suitable drop-out plates and for the OLE1::TDH3p-M.alpina ole1 genotype by colony-PCR, using the primers 5´ ATGGCAACTCCTCTTCCCCCCTCC 3´ and 5' AGACATTGAAATCCAAAGAAGACTGAAGG 3'. Mating type was scored by replica-plating to a lawn of cells with either a or alpha mating type, incubating at 30°C to allow mating, replica-plating to sporulation medium, and visualizing sporulation by illuminating plates under a 302 nm UV-light source. The haploid strains with the mating types MATa trp1 OLE1::TDH3p-M.alpina ole1 and MATa ura3 trp1 OLE1:TDH3p-M.alpina ole1 were named FS01315 and FS01316, respectively.

Example 8

Expression of the pathway to arachidonic acid in combination with a heterologous delta-9 desaturase in yeast

Yeast strains, in which the native OLE1 was replaced with ole1 from M. alpina (Example 7) and containing the appropriate genetic markers, were transformed with the vectors described in Examples 2, 3, 4, and 5, separately or in combination. Transformants were selected on medium lacking uracil and tryptophane and streak purified on the same medium.

S. cerevisiae strain FS01315 (MATa trp1 OLE1::TDH3p-M.alpina ole1) was transformed separately with the vector pESC-TRP-delta-12 (Example 2), yielding the strain FS01326, and with pESC-TRP-delta-12 delta-6 (Example 3), resulting in the strain FS01327. *S. cerevisiae* strain FS01316 (MATa trp1 ura3 OLE1::TDH3p-M.alpina ole1) was co-transformed with pESC-TRP-delta-12 delta-6 and pESC-URA-elo (Example 4), and the transformed strain was named FS01328. The same strain was also co-transformed with pESC-TRP-delta-12 delta-6 and pESC-URAelo-delta-5 (Example 5), resulting in the strain FS01329.

Example 9

Fermentation with recombinant yeast strains in shake flasks

Single yeast colonies were inoculated into 100 ml minimal medium (5 g/L glucose, 20 g/L galactose, 15 g/L (NH4)2SO4, 1 g/L MgSO4·7H2O, 14.4 g/L KH2PO4, 1mL/L vitamin solution, 1 mL/L trace metal solution, pH 6.5) in 500 ml baffled shake flasks. The vitamin solution contained: 50 mg/L biotin, 1 g/L calcium panthotenate, 1 g/L nicotinic acid, 25 g/L myo-inositol, 1 g/L thiamine HCl, 1 g/L pyridoxal HCl and 0.2 g/L para-aminobenzoic acid, while the trace metal solution contained: 15 g/L EDTA, 4.5 g/L $ZnSO_4 \cdot 7H_2O$, 1 g/L $MnCl_2 \cdot 2H_2O$, 0.3 g/L $CoCl_2 \cdot 6H_2O$, 0.4 g/L $Na_2MoO_4 \cdot 2H_2O$, 4.5 g/L $CaCl_2 \cdot 2H_2O$, 3 g/L $FeSO_4 \cdot 7H_2O$, 1 g/L H_3BO_3 and 0.1 g/L KI. For the ura3 strain FS01309, 100 ml/L of an amino acid cocktail (0.5 g/L histidine, 0.5 g/L tryptophane, 0.5 g/L uracil, 0.5 g/L leucine) was added to the medium by filtering through a 0.22 μm sterile filter. The cultures were incubated shaking (150 rpm) at 18 or 30°C for 96 or 72 hours, respectively. Following incubation, the biomass was collected by filtration and the lipid composition was analyzed as described in Example 11.

Example 10

Fermentation with recombinant yeast strains in fermenters

The recombinant yeast strains can be grown in fermenters operated as batch, fed-batch or chemostat cultures.

Batch and Fed-batch cultivations

For the precultures, single yeast colonies are inoculated into 100 ml minimal medium in 500 ml baffled shake flasks as described in example 9 and incubated shaking (150 rpm) at 30°C. Exponentially growing precultures are used for inoculation of batch cultivations at a starting concentration of 1 mg DW/L. Batch cultivations can be carried out in laboratory fermenters (e.g. B. Braun Biotech, Melsungen, Germany) with a working volume of 2 L. For the cultivations can be used a defined medium containing: 40 g/L glucose or galactose; 5.0 g/L $(NH_4)_2SO_4$; 3.0 g/L KH_2PO_4; 0.5 g/L $MgSO_4 \cdot 7H2O$ is used; and trace metals and vitamins as described in example 9. Antifoam (300 μl/L, Sigma A-8436) is added to avoid foaming. The choice of carbon source is dependent on the promoters chosen for heterologous expression; for example,

if the GAL1/GAL10 promoters are used, galactose is used as carbon source and if constitutive yeast promoters are used, glucose is generally chosen as carbon-source. The carbon source should be autoclaved separately from the minimal medium and afterwards added to the fermenter. Also, the vitamin and trace metal solutions are added to the fermenter by sterile filtration following autoclaving and cooling of the medium. Cultivations are performed at a fixed temperature, e.g. 18°C or 30°C, with a stirrer speed of 600 rpm and with 1 vvm (volume air per volume liquid per minute) aeration. The pH is controlled at 5.0 by automatic addition of 4 M KOH. The bioreactors are fitted with cooled condensers, and the off-gas can be led to a gas analyser (INNOVA, Ballerup, Denmark) to measure the off-gas content of CO_2.

Chemostat cultures

In chemostat cultures the cells can be grown in, for example, 1-L working-volume Applikon laboratory fermentors. In brief, the cultures are fed with a defined medium containing glucose or galactose as the growth-limiting nutrient (same medium as for the batch fermentations). The dilution rate (which equals the specific growth rate) in a steady-state culture can be set at different values, e.g. at 0.050 h^{-1}, 0.10 h^{-1}, 0.15 h^{-1}or 0.20 h^{-1}. The temperature is set at a fixed value, e.g. 18°C or 30°C, and the culture pH is set to 5.0. Aerobic conditions are maintained by spraying the cultures with air (e.g. 0.5 L/min). The dissolved-oxygen concentration, which is continuously monitored, e.g. with an Ingold model 34 100 3002 probe, is kept above 50% of air saturation.

Example 11

Analysis of PUFAs as methyl esters

Cells are grown in 100 ml shake flasks using the minimal medium as described in example 9 until the carbon source is exhausted. The biomass is separated through centrifugation at 3000 rpm, and the lipids are extracted using 30 ml of a chloroform/methanol mixture (2:1, v:v) overnight. In the next step, the sample is filtrated, the solvent solution is washed with 6 ml NaCl and the sample is dried over nitrogen. To the lipids, 2 ml toluene and 2 ml 1% sulphuric acid in methanol are added, and the sample is left at 50°C overnight for transesterification of the lipids. The sample is washed with 5 ml NaCl solution and vortexed. Extraction of methyl esters start with the

addition of 5 ml hexane, then the sample is vortexed and the upper hexane phase is removed. Another 5 ml hexane is added and the extraction is continued. 4 ml sodium carbonate is added to the hexane, the sample is vortexed and the phases are seperarted through centrifugation at 2000 rpm for 2 min. The sample is dried using anhydrous sodium sulphate and the solution is filtrated and dried over nitrogen. To the dried sample 0.5 ml of hexane is added and the sample is ready for determination of methyl esters, which is conducted using a gas chromatograph coupled to mass selective detector (GC/MS). The GC/MS is a Hewlett Packard HP G1723A, gas chromatograph-quadruple mass selective detector (EI) operated at 70 eV. The column is a JW-1701, 30 m, 250 µm i.d., 0.15µm film thickness. The MS is operated in SCAN Mode. The oven temperature is initially 170°C and in the following risen to 220°C at 4°C/min. The final temperature is held for 3 min. The flow through the column is 1 ml He/min. Injection volumes are 5 µl. The injector is driven at split of 100:1splitless for all analyses. The temperature of the inlet is 300°C, the interface temperature 230 °C, and the quadropule temperature 105°C. Detected fatty acid methyl esters are confirmed with the 1998 NIST Mass Spectral Database, and retention times are confirmed with standard fatty acid methyl esters. A typical gas chromatogram is shown in figure 11:

Example 12

Fatty acid compositions of recombinant yeast strains

The recombinant yeast strain FS01324, expressing the *M. alpina* genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase, and the recombinant yeast strain FS01329, which in addition to the mentioned *M. alpina* genes also expresses the *M. alpina ole1* gene, were cultivated as described in example 9 and the fatty acid composition was analyzed as described in example 11. A wild-type strain (FS01201) of the same genetic background was also included in the analysis as a reference. Strains FS01329 and FS01201 were cultivated at 30°C, while FS01324 was cultivated at 17°C.

The results of the analysis (Table 2) show that arachidonic acid was produced in both recombinant strains. As expected, the percentage of arachidonic acid was higher in FS01329 than in FS01324, i.e approximately two times higher. Furthermore, the ratio of stearic acid (18:1) to palmitoleic acid (16:1) was dramatically increased in the *ole1* expressing strain FS01329.

Table 2. Fatty acid composition (% of total fatty acid) of the recombinant yeast strains FS01324 and FS01329, and of the wild-type strain FS01201.

Fatty acid	FS01201	FS01324	FS01329
12:0	1.9	2.5	0.51
16:0	21.8	13.3	29.1
16:1	46.5	48.4	5.8
18:0	7.8	4.5	4.1
18:1	20.0	12.6	23.8
18:2	-	9.7	19.8
18:3	-	4.0	10.2
20:3	-	-	1.3
20:4	-	0.8	1.7
Other	2.1	4.2	3.7

Example 14

Integration of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase into the yeast genome

The genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase, isolated according to Example 1, are each placed downstream of a separate strong, constitutive yeast promoter (e.g. TDH3 promoter, ADH1 promoter, GPD promoter or TPI promoter) on an a single plasmid. The plasmid also contains a target sequence for integration into the yeast genome by homologous recombination and the *K. lactis URA3* gene flanked by direct repeats. The target sequence is engineered to contain a unique restriction site to allow linearization of the plasmid.

Suitable yeast strains (e.g. a strain with the genotype MATa ura3 and a strain with the genotype MATa *ura3* OLE1::TDH3p-M. alpina ole1) are transformed with the linearized plasmid, and transformants are selected on medium lacking uracil. After streak purification on the same medium, pop-out of the *K. lactis URA3* marker is selected for on medium containing 5-FOA. Correct integration of the plasmid is verified by PCR and sequencing of the modified region. To introduce desired genetic features into the resulting strain, it is crossed to a suitable yeast strain of opposite mating type. Following selection of diploids, sporulation and dissection, the novel haploid strains are scored by the methods described in Example 7.

Example 15

Cloning of a delta-5 elongase into a yeast expression vector

The mouse gene Ssc2 encodes a protein with sequence homology to a fatty acid elongase from yeast, ELO1p (Tvrdik et al. 2000). Expression of the gene in Human Embryonic Kidney 293 Cells, followed by in vitro assays of proteins extracted from these cells, has shown that the Ssc2 gene product can elongate 20:4 (n-6) and 20:5 (n-3), i.e. arachidonic acid and eicosapentaenoic acid (Moon et al. 2001).

Mouse Ssc2 (Tvrdik et al. 2000, Moon et al. 2001) was isolated by PCR using Mouse liver cDNA (Quick-Clone cDNA, Clontech) as template and the primers 5´GAAGATCTCCACCATGGAGCAGCTGAAGGCCTTTGATAATG 3´ and 5´CCTTAATTAAGGCTTATTGAGCCTTCTTGTCCGTCATGCCATTAGC 3´. These primers contained BglII and PacI restriction sites, allowing ligation of the BglII/PacI digested PCR fragment into BglII/PacI digested pESC-TRP vector, resulting in the vector pESC-TRP-delta-5elo.

Example 16

Cloning of a omega3 desaturase into a yeast expression vector

C. elegans fat1 (Spychalla et al. 1997) is amplified from a *C. elegans* cDNA library (Stratagene) using a gene-specific forward primer containing a KpNI restriction site and a gene-specific reverse primer containing a NheI restriction site. The PCR product is digested with KpNI/NheI and ligated into KpNI/NheI digested pESC-TRP-delta-5elo vector (Example 15), yielding the vector pESC-TRP-delta-5elo-omega3.

Example 17

Cloning of a delta-4 desaturase into a yeast expression vector

Thraustochytrium sp. ATCC 26185 is cultivated in a 500 ml shake flask containing 100 ml medium at room temperature with shaking. Following harvest of biomass, total RNA is isolated and used for cDNA preparation using Oligo(dT)12-18 as primer. Thraustochytrium Fad4 is amplified using the Thraustochytrium cDNA as template and

gene-specific primers containing suitable restriction sites. The Fad4 gene is then ligated into a suitable yeast expression vector (e.g. a high-copy vector with HIS or LEU selection and a galactose-inducible or constitutive promoter).

Example 18

Expression of the pathway to Docosahexaenoic acid in yeast

A yeast strain, containing genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase integrated in its genome (Example 14), is co-transformed with pESC-TRP-delta-5elo-omega-3 (Example 16) and an expression vector containing a gene encoding a delta-4 desaturase (Example 17).

Example 19

Integration of genes encoding delta-5 elongase, omega-3 desaturase and delta-4 desaturase into the yeast genome

Genes encoding delta-5 elongase, omega-3 desaturase and delta-4 desaturase are each placed downstream of a separate strong, constitutive yeast promoter (e.g. TDH3 promoter, ADH1 promoter, GPD promoter or TPI promoter) on a single plasmid. The plasmid also contains a target sequence for integration into the yeast genome by homologous recombination and the K. lactis *URA3* gene flanked by direct repeats. The target sequence is engineered to contain a unique restriction site to allow linearization of the plasmid. Suitable yeast strains are transformed with the linearized plasmid, and transformants are selected on medium lacking uracil. After streak purification on the same medium, pop-out of the *K. lactis URA3* marker is selected for on medium containing 5-FOA. Correct integration of the plasmid is verified by PCR and sequencing of the modified region. In order to introduce desired genetic features into the resulting strain, it is crossed to a suitable yeast strain of opposite mating type. Following selection of diploids, sporulation and dissection, the novel haploid strains are scored by the methods described in Example 7.

Example 20

Construction of vectors for PUFA production in E. coli

The genes needed for PUFA production (e.g. genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase for arachidonic acid production) are fused by PCR such that the stop and start codons overlap each other. Suitable primers are used, such that unique restriction sites are introduced at the 5´ end and 3´ end of the fusion product. The fusion product is ligated into an *E.coli* expression vector (e.g. pTXB1, pBR322 or a pUC vector) downstream of the promoter, resulting in an artifical operon. More genes (e.g. delta-5 elongase, delta-3 desaturase, delta-4 desaturase) can be added to the operon, again using a PCR-fusion approach followed by insertion at the 3´ end restriction site that was originally used for cloning of the cluster. Preferably, the expression system is based on a constitutive promoter, such as the bacteriophage gamma tandem promoter PR, PL or a strong constitutive *E.coli* promoter. Alternatively, the system is temperature-inducible, e.g. the bacteriophage gamma tandem promoter PR, PL is used in combination with the cI857 repressor, or IPTG-inducible, i.e the T7 promoter is used.

Example 21

Production of PUFAs in E. coli

An *E. coli* strain of appropriate genotype is transformed with an expression vector containing an artificial gene cluster with the genes required for production of a PUFA (Example 21) and recombinant cells are identified on selection medium, e.g. LB medium containing 5 mg/L ampicillin. Single colonies are inoculated into 100 ml medium, which can be 20 g/L glucose, 3 g/L KH_2PO_4, 7 g/L K_2HPO_4, 2 g/L $(NH_4)_2SO_4$ and 0.25 g/L $MgSO_4 \cdot 7H_2O$ supplemented with 5 $mg \cdot l^{-1}$ ampicillin, in a baffled 500 ml shake flask. The cultures are incubated with 200 rpm shaking at 37°C for 16-20 hours, until the carbon source is exhausted, and the biomass is harvested for analysis of fatty acid composition as described in example 11. If an IPTG-inducible promoter, such as the T7 promoter, is used, IPTG is added to to the medium at a final concentration of 0.01-1 mM.

Example 22

General molecular biology methods used in strain construction

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by: Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory: Cold Spring

Harbor, NY (1989). Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art as described by, e.g. Manual of Methods for General Bacteriology (Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E. W., Wood, W.A., Krieg, N.R., and Briggs, G., Eds.) American Society for Microbiology: Washington, D.C. (1994). All chemicals and reagents used for maintenance and growth of cells were obtained from Sigma, DIFCO Laboratories or GIBCO/BRL unless specified otherwise. Restriction enzymes and DNA ligase was purchased from New England Biolabs. All PCR reactions were carried out using the Phusion polymerase (Finnzymes). Oligonucleotides and sequencing services were purchased from MWG Biotech, Ebersberg, Germany. Purification of DNA fragments was carried out using GFX-columns (Amersham) or the QiaexII purification kit (Qiagen).

E. coli DH5α cells were made competent by the Inoue method as described in Sambrook et al., supra. *E. coli* cells were typically grown at 37°C in Luria Bertani (LB) medium, supplied with 50 mg/l ampicillin where necessary.

Yeast cells were typically grown at 30°C in YPD medium or synthetic complete drop-out medium, and were made competent by a LiAc-based method (Sambrook et al., supra). Genomic modifications (overexpression and deletion of genes, integration of heterologous genes) were performed by means of homologous recombination using PCR-generated targeting substrates and the *K.lactis URA3* gene as a selectable marker, essentially as described in Erdeniz, N., Mortensen, U.H., Rothstein, R. (1997) Genome Res. 7:1174-83. Information on primer design for fusion PCR can be found in the same publication. Generally, fusion of DNA fragments was made possible by using primers with appropriately designed 5' overhangs for amplification of the original DNA fragments. In all cases, PCR-generated fragments were excised from a 1% agarose gel and purified before proceeding with fusion PCR. Transformants were generally selected on –URA plates, and pop-out of the *K.lactis URA3* marker gene was selected for by plating on 5-FOA medium (5-fluoroorotic acid, 750 mg/l). Correct integration of promoters and heterologous genes was verified by PCR, always using one primer annealing to a sequence outside of the target sequence for integration and one primer annealing inside the sequence to be integrated. Gene deletions were also verified by PCR, using primers on both sides of the deleted gene. Generally, PCR-verification of genomic modifications was performed by means of colony-PCR. For colony-PCR, a small amount of cells was dispersed in 10 µl H_2O and was placed at -80°C for approximately 30 min, followed by 15 min. incubation at 37°C. The cell suspension was then used as template for PCR.

Methods for combining genetic features by crossing of strains used in Examples are well known and are, e.g., described in: Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1997). Typically, strains of opposite mating types were allowed to mate, diploids were selected and transferred to sporulation medium (20 g/l potassium acetate, 1 g/l glucose, 2.5 g/l yeast extract, pH 7.0) and were allowed to sporulate at 30°C for approximately 3 days. The asci were dissected on a YPD plate using a Singer MSM microscope and micromanipulator dissection microscope. The mating types of the resulting tetrads were scored by replica-plating to a lawn of cells with either a or alpha mating type, incubating at 30°C to allow mating, replica-plating to sporulation medium, and visualizing sporulation by illuminating plates under a 302 nm UV-light source. Auxotrophic markers were scored by replica plating to drop-out plates. Genetic modifications that could not be scored by phenotype were scored by colony-PCR. In general, the same primer sets that were used for verification of genomic integrations or knockouts were also used for colony-PCR scoring of tetrads (see above).

The genetic nomenclature used for describing the genotypes of the strains is as follows: Native yeast genes are written in capital letters, while deleted or mutated native yeast genes are written in small letters. Fungal genes are written in small letters, for example *M. alpina ole1*, *S. macrospora acl1*. Yeast promoters are indicated by a small p, for example *pADH1*, *pTDH3* for the *ADH1* and *TDH3* promoters. Overexpressions of native yeast genes by the promoter-replacement method are indicated by the promoter name followed by the gene name, for example *pADH1-FAS1*, *pTDH3-DGA1* for overexpression of *FAS1* with the *ADH1* promoter and overexpression of *DGA1* with the *TDH3* promoter. Disruption of native yeast genes are indicated by a double colon, for example *pox1::pTDH3-M. alpina ole1*, which means that the *POX1* gene has been disrupted and that the *TDH3* promoter and the *M. alpina ole1* gene has been integrated in its place. Plasmids are written in brackets.

Example 23

Construction of pWAD1 and pWAD2

The two vectors pWAD1 and pWAD2 were used as templates for PCR in the construction of gene-targeting substrates for overexpression of genes with the *ADH1* promoter. For construction of pWAD1 and pWAD2, the *ADH1* promoter (consisting of the 1467 bp immediately upstream of the start codon of the *ADH1* gene) was amplified from genomic yeast DNA using the primers 5´ AAATCGATAACCGCGGAGGGGGATCGAAGAAATGATGG 3´ and 5´ TTGGGCCCTTTCCCGGGTGTATATGAGATAGTTGATTGTATGC 3´. These primers introduced a *Cla*I and a *Sac*II restriction site at the 5' end of the promoter sequence, and *Xma*I and *Apa*I restriction sites at the 3' end. For construction of pWAD1, the ADH1 promoter fragment was digested with *Cla*I and *Apa*I and was introduced into ClaI/ApaI digested pWJ716. This resulted in a plasmid construct, where the ADH1 promoter was placed immediately downstream of *K. lactis URA3*. Absence of mutations in the *ADH1* promoter sequence was verified by sequencing of pWAD1. For construction of pWAD2, the *ADH1* promoter fragment was released from pWAD1 through digestion with *SacII* and *XmaI*. The fragment was purified and was introduced into SacII/XmaI digested pWJ716. This resulted in a plasmid construct, where the *ADH1* promoter was placed immediately upstream of *K. lactis URA3*. Plasmid maps of pWAD1 and pWAD2 are shown in Figure 13. The plasmid pWJ716, carrying the *K. lactis URA3* structural under the control of its native promoter and connected to its native terminator sequence, was a kind gift from Uffe H. Mortensen, Center for Microbial Biotechnology, Bio-Centrum DTU, Technical University of Denmark.

Example 24

Construction of pWJ716-TD1 and pWJ716-TD2

The two vectors pWJ716-TD1 and pWJ716-TD2 were used as templates for PCR in the construction of gene-targeting substrates for overexpression of genes with the *TDH3* promoter. For construction of pWJ716-TD1 and pWJ716-TD2, the *TDH3* promoter (consisting of the 1067 bp immediately upstream of the start codon of the *TDH3* gene) was amplified from genomic yeast DNA using the primers 5´TTGGGCCCTTTCCCGGGTTTTGTTTGTTTATGTGTG 3´and 5´AAATCGATAACCGCGGATGAAAGAAGCTTACCAG 3´. These primers introduced a *Cla*I and a *Sac*II restriction site at the 5' end of the promoter sequence, and *Xma*I and *Apa*I restriction sites at the 3' end. For construction of pWJ716-TD1, the *TDH3* promoter fragment was digested with *Cla*I and *Apa*I and was introduced into ClaI/ApaI digested pWJ716. This resulted in a plasmid construct, where the *TDH3* promoter was placed

immediately downstream of *K. lactis URA3*. Absence of mutations in the *TDH3* promoter sequence was verified by sequencing of pWJ716-TD1. For construction of pWJ716-TD2, the *TDH3* promoter fragment was released from pWJ716-TD1 through digestion with *SacII* and *XmaI*. The fragment was purified and was introduced into SacII/XmaI digested pWJ716. This resulted in a plasmid construct, where the *TDH3* promoter was placed immediately upstream of *K. lactis URA3*. Plasmid maps of pWJ716-TD1 and pWJ716-TD2 are shown in Figure 14.

Example 25

Overview of genetically modified yeast strains

A number of genetically modified yeast strains were constructed as described in the Examples. An overview of the strains mentioned in the Examples is given in Table 3. All modifications were made in the CEN.PK genetic background. The strains FS01267, FS01269 and FS01277 were obtained by crossing of the strains CEN.PK 110-10C and CEN.PK 113-6B, dissecting the asci of the resulting diploids and scoring the genotype of the resulting haploid strains.

Table 3. Overview of strains used or constructed in Examples. The table shows only genomic modifications; for strains expressing PUFA pathways from plasmids, see Table 4 and 11. SDG, Scientific research and Development GmbH, Oberusel, Germany; FS, Fluxome Sciences A/S

Strain	**Genotype**	**Source**
CEN.PK 113-7D	*MATa*	SDG
CEN.PK 113-5D	*MATa ura3*	SDG
CEN.PK 110-10C	*MATalpha his3*	SDG
CEN.PK 113-6B	*MATa ura3 trp1 leu2*	SDG
FS01267	*MATa trp1ura3*	FS
FS01269	*MATalpha trp1*	FS
FS01277	*MATa ura3 leu2 trp1*	FS
FS01309	*MATa ura3 ole1::pTDH3-M.alpina ole1*	Example 7
FS01316	*MATa ura3 trp1 ole1::pTDH3-M.alpina ole1*	Example 7
FS01351	*MATa ura3 pADH1-FAS1*	Example 26
FS01352	*MATa ura3 pADH1-FAS2*	Example 26
FS01342	*MATalpha trp1 pADH1-FAS1*	Example 26

FS01372	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2*	Example 26
FS01392	*MATa ura3 trp1 pADH1-FAS1* p*ADH1-FAS2 pTPI1-ACC1*	Example 27
FS01367	*MATa ura3 pox1::pTDH3-M.alpina ole1*	Example 28
FS01368	*MAT alpha ura3 trp1 pox1::pTDH3-M.alpina ole1*	Example 28
FS01344	*MATa ura3 pTDH3-DGA1*	Example 29
FS01370	*MATa ura3 trp1 pTDH3-DGA1*	Example 29
FS01425	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pox1::pTDH3-M.alpina ole1-pADH1-S. macrospora acl1-pTDH3- S. macrospora acl2*	Example 33
FS01395	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-GAT1*	Example 30
FS01394	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-SLC1*	Example 30
FS01393	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-YDR531W*	Example 30
FS01427	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pADH1-YBR159W*	Example 31
FS01440	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pADH1-TSC13*	Example 31
FS01254	*MATalpha ura3 gdh1::loxP gdh2::PGKp-GDH2-KanMX3*	Example 35
FS01398	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP gdh2::PGKp-GDH2-KanMX3*	Example 35
FS01419	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP glt1::PGKp-GLT1-KanMX3*	Example 35
FS01420	*MATalpha ura3 trp1 pADH1-FAS1 gdh1::loxP gln1::PGKp-GLN1-KanMX3*	Example 35
FS01437	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP glt1::PGKp-GLT1-KanMX3 gln1::PGKp-GLN1-KanMX3*	Example 35
FS01396	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pox1::pTDH3-M. alpina ole1*	Example 36
FS01408	*MATalpha ura3 trp1 pADH1-FAS1 pox1::pTDH3-M. alpina ole1*	Example 36

FS01423	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pTPI1-ACC1 pox1::pTDH3-M. alpina ole1*	Example 36
FS01444	*MATalpha ura3 trp1 leu2 pox1::pTDH3-M. alpina ole1*	Example 57
FS01460	*MATalpha ura3 trp1 leu2 pox1::pTDH3-M. alpina ole1 gpp1::pGAL1-S.kluyveri FAD3*	Example 61

Example 26

Overexpression of fatty acid synthase (FAS)

The two genes *FAS1* and *FAS2*, encoding the beta and alpha subunits of the yeast fatty acid synthase, respectively, were overexpressed with a strong yeast promoter. This was done by replacing the native *FAS1*- and *FAS2* promoters with the *ADH1* promoter, using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15). The two genes were overexpressed separately and the modifications were subsequently combined through crossing of strains. For each of the overexpressions, one part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *ADH1* promoter sequence and a target sequence corresponding to the beginning of the gene to be overexpressed. The second part of the bipartite substrate consisted of a target sequence upstream of the gene to be overexpressed, fused to the *ADH1* promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which the native promoter had been knocked out and replaced with two copies of the *ADH1* promoter sequence as a direct repeat on either side of the *K. lactis URA3* marker gene. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the *URA3* gene. This resulted in a strain, in which the native promoter had been replaced with the *ADH1* promoter.

The procedure was as follows:

For construction of part 1 of the bipartite gene targeting substrates (Figure 15), a fragment consisting of two thirds of *K. lactis URA3* (towards the 3' end) and the *ADH1* promoter was amplified from plasmid pWAD1. For overexpression of *FAS1*, the primer pair 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5'

TGGTCTTGTGGAGTAAGCGTCCATTGTATATGAGATAGTTGATTGTATGC 3' was used for this amplification and for overexpression of *FAS2*, the primer pair 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' TTCTTGCTCAACTTCCGGCTTCATTGTATATGAGATAGTTGATTGTATGC 3' was used. Furthermore, downstream target sequences, consisting of the beginning of *FAS1* and *FAS2*, respectively, were amplified from genomic yeast DNA by PCR using the primer pair 5' ATGGACGCTTACTCCACAAGACCATTAAC 3' and 5' TTGATATAGATCACGCAATTCTTCAAAGTAGTC 3' for the *FAS1* targeting sequence and the primer pair 5' ATGAAGCCGGAAGTTGAGCAAGAATTAGC 3' and 5' ACTTCTTCAACTTGTGAGCAACCAAAACG 3' for the *FAS2* targeting sequence. Finally, the *FAS1* and *FAS2* downstream targeting sequences were fused to the fragment consisting of two thirds of *K. lactis URA3* (towards the 3' end) and the *ADH1* promoter. For *FAS1*, the primer pair 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' TTGATATAGATCACGCAATTCTTCAAAGTAGTC 3' was used for the fusion reaction and for *FAS2*, the primer pair 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' ACTTCTTCAACTTGTGAGCAACCAAAACG 3' was used. The resulting fusion fragments 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*FAS1*) and 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*FAS2*) were part 1 of the bipartite targeting substrate used for *FAS1* and *FAS2* promoter replacement, respectively.

For construction of part 2 of the bipartite targeting substrate, a fragment consisting of the *ADH1* promoter and two thirds of *K.lactis URA3* towards the 5' end was first amplified by PCR using plasmid pWAD2 as template. The primers used for this amplification were 5' GGACGTTCCCTGTATGTACTAGGGGGATCGAAGAAATGATGG 3' and 5' GAGCAATGAACCCAATAACGAAATC 3'. Next, upstream targeting sequences were amplified from genomic yeast DNA using the primers 5' CCGCTGTACTATGCGGTCTCGTCC 3' and 5' AGTACATACAGGGAACGTCCGTATGCCAAAAATGCCAAAATGCC 3' for the *FAS1* upstream targeting sequence and 5' CAACTACAAGGAGGAGAATAAAGAGCAAGCC 3' and 5' AGTACATACAGGGAACGTCCAACGACAACAACAACGACTACAATGATGG 3' for the *FAS2* upstream targeting sequence. The upstream targeting sequences were then fused to the previously constructed p*ADH1*-5' 2/3 *K.lactis URA3* fragment. The primers used for the fusion reaction were 5' CCGCTGTACTATGCGGTCTCGTCC 3' and 5' GAGCAATGAACCCAATAACGAAATC 3' for *FAS1* and 5' CAACTACAAGGAGGAGAATAAAGAGCAAGCC 3' and 5' GAGCAATGAACCCAATAACGAAATC 3' for *FAS2*. The resulting fusion fragments UP(*FAS1*)-p*ADH1*-5' 2/3 *K. lactis URA3* and

UP(*FAS2*)-p*ADH1*-5' 2/3 *K. lactis URA3* were part 2 of the bipartite targeting substrate used for *FAS1* and *FAS2* promoter replacement, respectively.

For *FAS1* overexpression, the yeast strain CEN.PK 113-5D (MATa ura3) was transformed with the linear substrates UP(*FAS1*)-p*ADH1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*FAS1*). For FAS2 overexpression, the same parent strain was transformed with the linear substrates UP(*FAS2*)-p*ADH1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*FAS2*). Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strains had the genotypes *MATa ura3 pADH1-FAS1* and *MATa ura3* p*ADH1-FAS2* and were named FS01351 and FS01352, respectively. Correct integration of the *ADH1* promoter and absence of PCR-generated mutations were verified by sequencing of the modified regions in both strains.

To combine overexpression of *FAS1* and *FAS2* in one strain, the *FAS1* overexpressing mutant FS01351 (*MATa ura3 pADH1-FAS1*) was first crossed to the strain FS01269 (*MATalpha trp1*). Diploids were selected on medium lacking uracil and tryptophane and were then transferred on to sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads. Presence of the p*ADH1-FAS1* modification in the resulting haploid strains was determined by colony PCR, and remaining genetic features were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MATalpha trp1 pADH1-FAS1* was selected and named FS01342.

FS01342 (*MATalpha trp1 pADH1-FAS1*) was then crossed to FS01352 (*MATa ura3* p*ADH1-FAS2*) and diploids were selected on medium lacking uracil and tryptophane. Following transfer of the diploids to sporulation medium, asci were dissected into ascospore tetrads. Presence of the p*ADH1-FAS1* and p*ADH1-FAS2* modifications in the resulting haploid strains was determined by colony PCR, and remaining genetic features were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2* was selected and was named FS01372.

Example 27

Overexpression of ACC1

The yeast gene *ACC1*, encoding acetyl-CoA carboxylase, was overexpressed with a strong constitutive yeast promoter. This was done by replacing the native *ACC1* promoter with the *TPI1* promoter, using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15). One part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *TPI1* promoter sequence and a target sequence corresponding to the beginning of *ACC1*. The second part of the bipartite substrate consisted of a target sequence upstream of *ACC1*, fused to the *TPI1* promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which the native promoter had been knocked out and replaced with two copies of the *TPI1* promoter sequence as a direct repeat on either side of the *K. lactis URA3* marker gene. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the *URA3* gene. This resulted in a strain, in which the native *ACC1* promoter had been replaced with the *TPI1* promoter.

In order to construct part 1 of the bipartite substrate, two thirds (towards the 3´end) of *K. lactis ura3*was amplified from the plasmid pWJ716 using the primers 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' CTGGAATTCGATGATGTAGTTTCTGG 3'. Moreover, the *TPI1* promoter sequence was amplified from genomic yeast DNA using the primers 5' CTACATCATCGAATTCCAGCTACGTATGGTCATTTCTTCTTC 3' and 5' TTTTTGATTAAAATTAAAAAAACTTTTTAGTTTATGTATGTGTTTTTTG 3' and a downstream targeting sequence, consisting of the beginning of the *ACC1* gene (i.e., the first 553 bp of the gene) was amplified from genomic yeast DNA using the primers 5' AGTTTTTTTAATTTTAATCAAAAAATGAGCGAAGAAAGCTTATTCGAGTC 3' and 5' CACCTAAAGACCTCATGGCGTTACC 3'. These three fragments were fused to each other in two rounds of PCR. First, the *TPI1* promoter sequence was fused to the downstream targeting sequence, using the primers 5' CTACATCATCGAATTCCAGCTACGTATGGTCATTTCTTCTTC 3' and 5' CACCTAAAGACCTCATGGCGTTACC 3'. The resulting product was then fused to the fragment containing two thirds (towards the 3´end) of *K. lactis URA3*. The resulting fragment, 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*ACC1*) was part 1 of the bipartite gene targeting substrate.

In order to construct part 2 of the bipartite substrate, two thirds (towards the 5´end) of *K. lactis URA3* was amplified from the plasmid pWJ716 using the primers 5' CGGTCTGCATTGGATGGTGGTAAC 3' and 5' GAGCAATGAACCCAATAACGAAATC 3'. The *TPI1* promoter sequence was amplified from genomic yeast DNA using the primers 5' CTACATCATCGAATTCCAGCTACGTATGGTCATTTCTTCTTC 3' and 5' CACCATCCAATGCAGACCGTTTTAGTTTATGTATGTGTTTTTTG 3', and a target sequence upstream of *ACC1* was amplified from genomic DNA using primers 5' TGTTCTGCTCTCTTCAATTTTCCTTTC 3' and 5' CTGGAATTCGATGATGTAGTTTCTAATTTTCTGCGCTGTTTCG 3'. These three fragments were fused in two rounds of PCR. First, the upstream targeting sequence was fused to the TPI1 promoter sequence, using the primers 5' TGTTCTGCTCTCTTCAATTTTCCTTTC 3' and 5' CACCATCCAATGCAGACCGTTTTAGTTTATGTATGTGTTTTTTG 3'. The resulting fragment was then fused to the fragment containing two thirds (towards the 5' end) of *K. lactis URA3*, resulting in the fragment UP(*ACC1*)-p*TPI1*-5' 2/3 *K. lactis URA3*, which constituted part 2 of the bipartite gene targeting substrate.

Yeast strain FS01372 (*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2*) was transformed with the linear substrates UP(*ACC1*)-p*TPI1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*ACC1*). Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype *MATa ura3 trp1 pADH1-FAS1* p*ADH1-FAS2 pTPI1-ACC1* and was named FS01392. Correct integration of the *TPI1* promoter was checked by colony PCR.

Example 28

Integration of M. alpina ole1 at the POX1 locus in S. cerevisiae

The *M. alpina ole1* gene, encoding a delta-9 desaturase, was integrated into the genome of *S. cerevisiae* and was placed under the control of the yeast *TDH3* promoter. The *TDH3* promoter and the *M. alpina ole1* gene were integrated at the locus of *POX1*, encoding the first enzyme in the beta-oxidation pathway, resulting in knockout of this gene. The integration was carried out through homologous recombination using a bipartite gene targeting substrate (Figure 16). One part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *TDH3* promoter sequence, the *M.*

alpina ole1 gene and a target sequence downstream of *POX1*. The second part of the bipartite substrate consisted of a target sequence upstream of *POX1*, fused to the *TDH3* promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which *POX1* had been knocked out and replaced with two copies of the *TDH3* promoter sequence as a direct repeat on either side of the *K. lactis* URA3 marker gene and the *M. alpina ole1* gene immediately downstream of the second *TDH3* promoter repeat. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the URA3 gene. This resulted in a strain, in which the *POX1* gene had been replaced with the *M. alpina ole1* under the control of the *TDH3* promoter.

The procedure was as follows:

For construction of part 1 of the bipartite gene targeting substrates (Figure 16), a fragment consisting of two thirds of *K. lactis URA3* (towards the 3' end) followed by the *TDH3* promoter was amplified by PCR from plasmid pWJ716-TD1, using primers 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' GGGGGGAAGAGGAGTTGCCATTTTGTTTGTTTATGTGTG 3'. Furthermore, the *M. alpina* ole1 gene (SEQ ID NO 1), isolated as described in Example 1, was reamplified by PCR using the primers 5´ATGGCAACTCCTCTTCCCCCCTCC 3´ and 5´TTGTTATTGTAATGTGATACCTATTCGGCCTTGACGTGG 3´, and a targeting sequence downstream of *POX1* was amplified from genomic yeast DNA by PCR using the primers 5' GTATCACATTACAATAACAATTCCTTCGAACCCTCTGTTTTGC 3' and 5' TTAGAGCTTCATTCCAACAAGTGCC 3'. These three fragments were then fused by two rounds of PCR. First, the *M. alpina ole1* gene was fused to the downstream targeting sequence, using the primers 5´ATGGCAACTCCTCTTCCCCCCTCC 3´ and 5' TTAGAGCTTCATTCCAACAAGTGCC 3'. The resulting fragment was then fused to the fragment containing two thirds of *K. lactis URA3* (towards the 3' end) followed by the *TDH3* promoter. The resulting fragment, 3' 2/3 *K. lactis URA3*-p*TDH3*-M. *alpina ole1*-DOWN(*POX1*), constituted part 1 of the bipartite targeting substrate.

For construction of the second part of the bipartite substrate, a target sequence upstream of *POX1* was amplified by PCR using *S. cerevisiae* genomic DNA as template and the primers 5´ AATTCGGTAAATTCAATGGGTAGG 3´ and 5´

TTAGTACATACAGGGAACGTCCGTAAATATAGGGCTTAAAATGTGTCAGG 3´. Furthermore, a fragment consisting of the *TDH3* promoter followed by two thirds of *K.lactis URA3* (towards the 5′ end) was amplified by PCR using plasmid pWJ716-TD2 as template. The primers used for this amplification were 5′ GGACGTTCCCTGTATGTACTAAAAATGAAAGAAGCTTACCAG 3′ and 5′ GAGCAATGAACCCAATAACGAAATC 3′. These two fragments were then fused by PCR using the primers 5´ AATTCGGTAAATTCAATGGGTAGG 3´ and 5′ GAGCAATGAACCCAATAACGAAATC 3′, resulting in the fragment UP(*POX1*)-p*TDH3*-5′ 2/3 *K. lactis URA3*, which constituted part 2 of the bipartite targeting substrate.

The yeast strain CEN.PK 113-5D (*MATa ura3*) was transformed with the linear substrates UP(*POX1*)-p*TDH3*-5′ 2/3 *K. lactis URA3* and 3′ 2/3 *K. lactis URA3*-p*TDH3*-M. *alpina ole1*-DOWN(*POX1*) and plated out on medium lacking uracil. Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype *MATa ura3 pox1::pTDH3-M.alpina ole1* and was named FS01367. Correct integration of the *TDH3* promoter and *M.alpina ole1* and absence of PCR-generated mutations was verified by sequencing of the modified region.

To combine *pox1::pTDH3-M.alpina ole1* modification with the appropriate genetic markers, FS01367 (*MATa ura3 pox1::pTDH3-M.alpina ole1*) was crossed to FS01269 (*MATalpha trp1*). Diploids were selected on medium lacking uracil and tryptophane and were then transferred on to sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads. Presence of the *pox1::pTDH3-M.alpina ole1* modification in the resulting haploid strains was determined by colony PCR, and remaining genetic features were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MAT alpha ura3 trp1 pox1::pTDH3-M.alpina ole1* was selected and named FS01368.

Example 29

Overexpression of DGA1

The yeast gene *DGA1*, encoding diacylglycerol acyltransferase, was overexpressed with a strong constitutive yeast promoter. This was done by replacing the native *DGA1* promoter with the *TDH3* promoter, using a promoter-replacement method based on a bipartite

gene-targeting substrate (Figure 15). One part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *TDH3* promoter sequence and a downstream targeting sequence corresponding to the beginning of *DGA1*. The second part of the bipartite substrate consisted of a target sequence upstream of *DGA1*, fused to the *TDH3* promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which the native promoter had been knocked out and replaced with two copies of the *TDH3* promoter sequence as a direct repeat on either side of the *K. lactis* URA3 marker gene. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the URA3 gene. This resulted in a strain, in which the native *DGA1* promoter had been replaced with the *TDH3* promoter.

The procedure was as follows:

For construction of part 1 of the bipartite gene targeting substrates (Figure 15), a fragment consisting of two thirds of *K. lactis ura3* (towards the 3' end) followed by the *TDH3* promoter was amplified by PCR from plasmid pWJ716-TD1, using primers 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' TTTGTTTGTTTATGTGTGTTTATTCGAAACTAAG 3'. Furthermore, a downstream targeting sequence, corresponding to the beginning of DGA1, was amplified by PCR from genomic yeast DNA by PCR using the primers 5' CGAATAAACACACATAAACAAACAAAATGTCAGGAACATTCAATGATATA 3' and 5' GTTTTAAATTGACAGTTTTAATCAAACTTATAGGG 3'. These fragments were then fused by PCR using the primers 5´ CTTGACGTTCGTTCGACTGATGAGC 3´ and 5' GTTTTAAATTGACAGTTTTAATCAAACTTATAGGG 3'. The resulting fragment, 3' 2/3 *K. lactis URA3*-p*TDH3*- DOWN(*DGA1*), constituted part 1 of the bipartite targeting substrate.

For construction of the second part of the bipartite substrate, a target sequence upstream of *DGA1* was amplified by PCR using *S. cerevisiae* genomic DNA as template and the primers 5´ TTTTGGCTGTTGTTCCAGGTCGTAGG 3´ and 5´ AGTACATACAGGGAACGTCCGATAAACAGGAAAAAAAAAAAACTTTGGCG 3´. Furthermore, a fragment consisting of the *TDH3* promoter followed by two thirds of *K.lactis URA3* (towards the 5' end) was amplified by PCR using plasmid pWJ716-TD2 as template. The primers used for this amplification were 5' GGACGTTCCCTGTATGTACTAAAAATGAAAGAAGCTTACCAG 3' and 5'

GAGCAATGAACCCAATAACGAAATC 3'. These two fragments were then fused by PCR using the primers 5´ TTTTGGCTGTTGTTCCAGGTCGTAGG 3´ and 5' GAGCAATGAACCCAATAACGAAATC 3', resulting in the fragment UP(*DGA1*)-p*TDH3*-5' 2/3 *K. lactis URA3*, which constituted part 2 of the bipartite targeting substrate.

The yeast strain FS01202 (MATa ura3) was transformed with the linear substrates UP(*DGA1*)-p*TDH3*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*TDH3*- DOWN(*DGA1*) and plated out on medium lacking uracil. Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype *MATa ura3 pTDH3-DGA1* and was named FS01344. Correct integration of the *TDH3* promoter and absence of PCR-generated mutations was verified by sequencing of the modified region.

To combine *DGA1* overexpression with the appropriate genetic markers, FS01344 (*MATa ura3 pTDH3-DGA1*) was crossed to FS01269 (*MATalpha trp1*). Diploids were selected on medium lacking uracil and tryptophane and were then transferred on to sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads. Presence of the *pTDH3-DGA1* modification in the resulting haploid strains was determined by colony PCR, and remaining genetic features were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MATa ura3 trp1 pTDH3-DGA1* was selected and named FS01370.

Example 30

Overexpression of GAT1, SLC1 and YDR531W

The yeast genes *GAT1*, encoding glycerol-3-phosphate acyltransferase, *SLC1*, encoding 1-acyl-sn-gylcerol-3-phosphate acyltransferase, and YDR531W, putatively encoding pantothenate kinase, were all overexpressed with the strong constitutive *TPI1* promoter using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15). For each of the overexpressions, the first part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *TPI1* promoter sequence and a target sequence corresponding to the beginning of the gene to be overexpressed. The second part of the bipartite substrate consisted of a target sequence upstream of the gene to be overexpressed, fused to the *TPI1* promoter sequence and two

thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which the native promoter had been knocked out and replaced with two copies of the *TPI1* promoter sequence as a direct repeat on either side of the *K. lactis URA3* marker gene. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5-FOA. In the pop-out recombinants, the native promoters had thus been replaced with the *TPI1* promoter, resulting in overexpression of *GAT1*, *SLC1* and *YDR531W*, respectively.

In order to construct part 1 of the bipartite substrate, two thirds (towards the 3´end) of *K. lactis URA3* was amplified from the plasmid pWJ716 using the primers 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' CTGGAATTCGATGATGTAGTTTCTGG 3'. Moreover, the *TPI1* promoter sequence was amplified from genomic yeast DNA using the primers 5' CTACATCATCGAATTCCAGCTACGTATGGTCATTTCTTCTTC 3' and 5' TTTTTGATTAAAATTAAAAAAACTTTTTAGTTTATGTATGTGTTTTTTG 3'. Downstream target sequences, consisting of the beginning of *GAT1*, *SLC1* and *YDR531W*, respectively, were then amplified from genomic yeast DNA. The primer pair 5' AGTTTTTTTAATTTTAATCAAAAAATGTCTGCTCCCGCTGCC 3' and 5' AACCTTTTCGTAAAGTTCACTGG 3' was used for amplification of the *GAT1* downstream targeting, the primer pair 5' AGTTTTTTTAATTTTAATCAAAAAATGAGTGTGATAGGTAGGTTCTTG 3' and 5' AGGAAAAACCCATAGAGCACG 3' was used for amplification of the *SLC1* targeting sequence, and the primer pair 5' AGTTTTTTTAATTTTAATCAAAAAATGCCGCGAATTACTCAAG 3' and 5' GACTAGAAGGTATGGGTAGATAGCC 3' was used for amplification of the *YDR531W* targeting sequence. Each of the *GAT1, SLC1* and *YDR531W* downstream target sequences were then fused to the TPI promoter by PCR, using the forward primer 5' CTACATCATCGAATTCCAGCTACGTATGGTCATTTCTTCTTC 3' and the reverse primers 5' AACCTTTTCGTAAAGTTCACTGG 3' (for fusion to the *GAT1* targeting sequence), 5' AGGAAAAACCCATAGAGCACG 3' (for fusion to the *SLC1* targeting sequence) or 5' GACTAGAAGGTATGGGTAGATAGCC 3' (for the *YDR531W* targeting sequence). Finally, the resulting fusion fragments were fused to the fragment consisting of two thirds (towards the 3´end) of *K. lactis URA3* by PCR using the forward primer 5' CTTGACGTTCGTTCGACTGATGAGC 3' and the reverse primers 5' AACCTTTTCGTAAAGTTCACTGG 3' (for fusion to the *GAT1* targeting sequence), 5' AGGAAAAACCCATAGAGCACG 3' (for fusion to the *SLC1* targeting sequence) or 5'

GACTAGAAGGTATGGGTAGATAGCC 3' (for the *YDR531W* targeting sequence). The resulting fragments, 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*GAT1*), 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*SLC1*) and 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*YDR531W*) constituted part 1 of the bipartite gene targeting substrate used for *GAT1* and *SLC1* overexpression, respectively.

In order to construct part 2 of the bipartite substrate, two thirds (towards the 5´end) of *K. lactis URA3* was amplified from the plasmid pWJ716 using the primers 5' CGGTCTGCATTGGATGGTGGTAAC 3' and 5' GAGCAATGAACCCAATAACGAAATC 3', and the *TPI1* promoter sequence was amplified from genomic yeast DNA using the primers 5' CTACATCATCGAATTCCAGCTACGTATGGTCATTTCTTCTTC 3' and 5' CACCATCCAATGCAGACCGTTTTAGTTTATGTATGTGTTTTTTG 3'. Furthermore, target sequences upstream of *GAT1*, *SLC1* and *YDR531W* were amplified from genomic yeast DNA by PCR using the primer pair 5' GGTAAAGAAAACTACAAATCTGGG 3' and 5' CTGGAATTCGATGATGTAGAAGCTGCCACTTCTTCAGGG 3' for the *GAT1* target sequence, the primer pair 5' TTGCTTTAAACATCTGTCCAAGAC 3' and 5' CTGGAATTCGATGATGTAGCCTTCACCTTAAACCCTTCC 3' for the *SLC1* target sequence, and the primer pair 5' TGTCTTCCTATTTTCTCTGACCC 3' and 5' CTGGAATTCGATGATGTAGTCGCATGCACTCAATTGG 3' for the *YDR531W* target sequence. Each of the *GAT1, SLC1* and *YDR531W* upstream target sequences were then fused to the TPI promoter by PCR, using the reverse primer 5' CACCATCCAATGCAGACCGTTTTAGTTTATGTATGTGTTTTTTG 3' and the forward primers 5' GGTAAAGAAAACTACAAATCTGGG 3' (for fusion to the *GAT1* targeting sequence) 5' TTGCTTTAAACATCTGTCCAAGAC 3' (for fusion to the *SLC1* targeting sequence), or 5' TGTCTTCCTATTTTCTCTGACCC 3' (for fusion to the *YDR531W* target sequence). Finally, the resulting fusion fragments were fused to the fragment consisting of two thirds (towards the 5´end) of *K. lactis URA3* by PCR using the reverse primer 5' GAGCAATGAACCCAATAACGAAATC 3' and the forward primers 5' GGTAAAGAAAACTACAAATCTGGG 3' (for fusion to the *GAT1* targeting sequence), 5' TTGCTTTAAACATCTGTCCAAGAC 3' (for fusion to the *SLC1* targeting sequence), or 5' TGTCTTCCTATTTTCTCTGACCC 3' (for fusion to the *YDR531W* target sequence). The resulting fragments, UP(*GAT1*)-p*TPI1*-5' 2/3 *K. lactis URA3,* UP(*SLC1*)-p*TPI1*-5' 2/3 *K. lactis URA3* and UP(*YDR531W*)-p*TPI1*-5' 2/3 *K. lactis URA3* constituted part 2 of the bipartite gene targeting substrate used for *GAT1, SLC1* and *YDR531W* overexpression, respectively.

For *GAT1* overexpression, the yeast strain FS01372 (*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2*) was transformed with the linear substrates UP(*GAT1*)-p*TPI1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*GAT1*). Similarly, for *SLC1* overexpression, FS01372 was transformed with the linear substrates UP(*SLC1*)-p*TPI1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*SLC1*) and for *YDR531W* overexpression, FS01372 was transformed with the linear substrates UP(*YDR531W*)-p*TPI1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*TPI1*-DOWN(*YDR531W*). Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strains had the genotypes *MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-GAT1*, *MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-SLC1*, and *MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1- YDR531W* and were named FS01395, FS01394 and FS01393, respectively. Correct integration of the *TPI1* promoter was verified by colony-PCR in all strains.

Example 31

Overexpression of YBR159W and TSC13

The yeast genes *YBR159W*, encoding beta-ketoacyl-CoA synthase, and *TSC13*, encoding trans-2-enoyl-CoA reductase, were both overexpressed with the strong constitutive *ADH1* promoter using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15). For each of the overexpressions, the first part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *ADH1* promoter sequence and a target sequence corresponding to the beginning of the gene to be overexpressed. The second part of the bipartite substrate consisted of a target sequence upstream of the gene to be overexpressed, fused to the *ADH1* promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which the native promoter had been knocked out and replaced with two copies of the *ADH1* promoter sequence as a direct repeat on either side of the *K. lactis URA3* marker gene. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5-FOA. In the pop-out recombinants, the native promoters had thus been replaced with the *ADH1* promoter, resulting in overexpression of *YBR159W* and *TSC13*, respectively.

The procedure was as follows:

For construction of part 1 of the bipartite gene targeting substrates (Figure 15), a fragment consisting of two thirds of *K. lactis URA3* (towards the 3' end) and the *ADH1* promoter was amplified from plasmid pWAD1 using the primers 5´ CTTGACGTTCGTTCGACTGATGAGC 3´ and 5' TGTATATGAGATAGTTGATTGTATGC 3'. Furthermore, downstream target sequences, consisting of the beginning of *YBR159W* and *TSC13*, respectively, were amplified by PCR from genomic yeast DNA using the primer pair 5' TACAATCAACTATCTCATATACAATGACTTTTATGCAACAGCTTCAAGAG 3' and 5' GACCAACATTATTGACCAAAACGG 3' for the *YBR159W* targeting sequence and the primer pair 5' GCATACAATCAACTATCTCATATACAATGCCTATCACCATAAAAAGCC 3' and 5' GGAAGCCGTAGCCAAAGTAACC 3' for the *TSC13* targeting sequence. Finally, the *YBR159W* and *TSC13* downstream targeting sequences were fused to the fragment consisting of two thirds of *K. lactis URA3* (towards the 3' end) and the *ADH1* promoter by PCR. For *YBR159W*, the primer pair 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' GACCAACATTATTGACCAAAACGG 3' was used for the fusion reaction and for *TSC13*, the primer pair 5' CTTGACGTTCGTTCGACTGATGAGC 3' and 5' GGAAGCCGTAGCCAAAGTAACC 3' was used. The resulting fusion fragments 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*YBR159W*) and 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*TSC13*) were part 1 of the bipartite targeting substrate used for *YBR159W* and *TSC13* promoter replacement, respectively.

For construction of part 2 of the bipartite targeting substrate, a fragment consisting of the *ADH1* promoter and two thirds of *K.lactis URA3* towards the 5' end was first amplified by PCR using plasmid pWAD2 as template. The primers used for this amplification were 5' GGACGTTCCCTGTATGTACTAGGGGGATCGAAGAAATGATGG 3' and 5' GAGCAATGAACCCAATAACGAAATC 3'. Next, upstream targeting sequences were amplified from genomic yeast DNA using the primer pair 5' GAAAAAATCATTGGATGCCC 3' and 5' AGTACATACAGGGAACGTCCAACGCTTTTATTCGTGAAATCTC 3' for the *YBR159W* upstream targeting sequence and the primer pair 5' GTTATTGAAAGCAATGGGCAAC 3' and 5' AGTACATACAGGGAACGTCCAATTCAAAATATGTATCTCTCTC 3' for the *TSC13* upstream targeting sequence. The upstream targeting sequences were then fused to the previously constructed p*ADH1*-5' 2/3 *K.lactis URA3* fragment. The reverse primer used for the fusion reactions was 5' GAGCAATGAACCCAATAACGAAATC 3' and the forward primers were 5' GAAAAAATCATTGGATGCCC 3' (for the *YBR159W* target sequence) or and 5' GTTATTGAAAGCAATGGGCAAC 3' (for the *TSC13* target sequence). The resulting fusion

fragments UP(*YBR159W*)-p*ADH1*-5' 2/3 *K. lactis URA3* and UP(*TSC13*)-p*ADH1*-5' 2/3 *K. lactis URA3* were part 2 of the bipartite targeting substrate used for *YBR159W* and *TSC13* promoter replacement, respectively.

For *YBR159W* overexpression, the yeast strain FS01372 (*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2*) was transformed with the linear substrates UP(*YBR159W*)-p*ADH1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*YBR159W*). Similarly, for *TSC13* overexpression, FS01372 was transformed with the linear substrates UP(*TSC13*)-p*ADH1*-5' 2/3 *K. lactis URA3* and 3' 2/3 *K. lactis URA3*-p*ADH1*- DOWN (*TSC13*). Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strains had the genotypes *MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pADH1-YBR159W* and *MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pADH1-TSC13* and were named FS01427 and FS01440, respectively. Correct integration of the *ADH1* promoter was verified by colony-PCR in both strains.

Example 32

Isolation of genes encoding subunits of a fungal ATP:citrate lyase

Fungal ATP:citrate lyases consist of two subunits, encoded by separate genes. In the oleaginous yeast *Sordaria macrospora* these two subunits are encoded by the genes *acl1* and *acl2* (Minou et al. 2000 Curr Genet 37:189-193). The coding sequences of *S. macrospora acl1* and *acl2* were isolated by PCR from first strand cDNA from *S. macrospora* CBS 957.73 as template. The defined primers used for the amplification were designed to match the published coding sequences of *acl1* (SEQ ID NO 80) and *acl2* (SEQ ID NO 82). The procedure was as follows:

S. macrospora CBS 957.73 was cultivated in 100 ml YEPA medium (1% yeast extract, 2% peptone and 2% acetate, pH 6.0) shaking at 100 rpm at room temperature for 4 days. Biomass was collected by filtration and total RNA was isolated using Trizol reagent (Gibco BRL). 5 µg of RNA was used for reverse transcription (Superscript II RT, Invitrogen) using Oligo(dT)12-18 as primer. After first strand cDNA sythesis, complementary RNA was removed by RNAse digestion. The cDNA was then used as template for PCR (Phusion enzyme, Finnzymes) using the following primers:

5′ GCATACAATCAACTATCTCATATACAATGCCTTCCGCAACTAGCACC 3′ and 5′ TTGTTATTGTAATGTGATACTTAAATTTGGACCTCAACACGACC 3′ for *acl1*; 5′ TTAGGCCTGGAACTCCACCGCAC 3′ and 5′ CGAATAAACACACATAAACAAACAAAATGTCTGCGAAGAGCATC 3′ for acl2. The resulting fragments of the expected sizes were excised from an agarose gel and purified using GFX-columns (Amersham).

Example 33

Integration of genes encoding subunits of a fungal ATP:citrate lyase into the genome of S. cerevisiae

The strategy used for integrating the two subunits of ATP:citrate lyase (*acl1* and *acl2*) is shown in Figure 17. As parent strain for integration into the genome, FS01396, a strain that carries the *M. alpina ole1* gene integrated at the *POX1* locus, was chosen. The *acl1* and *acl2* genes were integrated downstream of the *M. alpina ole1* gene in this strain, using a gene targeting substrate consisting of four linear fragments: ACL-F1, ACL-F2, ACL-F3 and ACL-F4. ACL-F1 contained the upstream targeting sequence for directing the substrate to the correct position in the genome, in addition to the CYC terminator sequence in forward orientation and the ADH1 terminator sequence in reverse orientation. ACL-F1 additionally contained a 3′ sequence of 20 bp which was identical to the 5′ end of ACL-F2. ACL-F2 contained the *acl2* gene in reverse orientation under the control of the TDH3 promoter, fused to the ADH1 promoter in forward orientation. ACL-F2 additionally contained a 3′ sequence of 20 bp that was identical to the 5′ end of ACL-F3. ACL-F3 consisted of the complete *K. lactis URA3* marker gene in forward orientation followed by the ADH1 promoter sequence in forward orientation. ACL-F4 consisted of the acl1 gene in forward orientation, fused to the downstream targeting sequence used for directing the substrate to the correct position in the genome. In addition, ACL-F4 contained a 5′ sequence of 20 bp that was identical to the 3′ end of ACL-F3.

The overlapping sequences at the 3′ end of ACL-F1 and 5′ end of ACL-F2, the 3′ end of ACL-F2 and 5′ end of ACL-F3, and between the 3′ end of ACL-F3 and the 5′ end of ACL-F4 allowed assembly of the complete gene targeting substrate *in vivo* by the homologous recombination mechanisms of *S. cerevisiae*. Thus, FS01396 was transformed with the four linear substrates ACL-F1, ACL-F2, ACL-F3 and ACL-F4, and integration of the complete gene targeting substrate at the intended genomic location was selected for by

plating out the transformed on medium lacking uracil. This resulted in a strain in which the complete targeting substrate was integrated immediately downstream of *M. alpina ole1*, in a way so that the *M. alpina ole 1* was now connected to the *CYC1* terminator. Moreover, the *acl2* gene was placed under the control of the *TDH3* promoter and was connected to the *ADH1* terminator sequence, all in reverse direction. The marker gene *K. lactis URA3* was integrated in forward direction and was flanked by the *ADH1* promoter as a direct repeat on either side of the marker. Finally, the *acl1* gene was placed under the control of the *ADH1* promoter and immediately upstream of the *POX1* terminator sequence, corresponding to the downstream targeting sequence (Figure 17). The *K. lactis URA3* marker gene was then looped out by replating transformants on 5-FOA medium.

The procedure was as follows:

For construction of the fragment ACL-F1, an upstream targeting sequence, corresponding to the 5' end of *M. alpina ole1*, was first amplified by PCR using genomic DNA as template and the primers 5' CAACGCTATCCGCTTTTACCAGT 3' and 5' CTATTCGGCCTTGACGTGGTCAGTGC 3'. Furthermore, a fragment containing the CYC1 and ADH1 terminators in reverse orientation was amplified by PCR using plasmid p300 as template and using the primers 5' GCACTGACCACGTCAAGGCCGAATAGCCGCTCTAACCGAAAAGGAAGG 3' and 5' GTGCGGTGGAGTTCCAGGCCTAACGAATTTCTTATGATTTATGATTTTTA 3'. The two fragments were then fused by PCR using the primers 5' CAACGCTATCCGCTTTTACCAGT 3' and GTGCGGTGGAGTTCCAGGCCTAACGAATTTCTTATGATTTATGATTTTTA 3', resulting in the fragment ACL-F1.

For construction of the fragment ACL-F2, a fragment containing the TDH3 promoter in reverse direction and the ADH1 promoter in forward direction was amplified by PCR using as template the plasmid pESC-URA-elo-delta-5-AT (Example 34) and using the primers 5' TTTGTTTGTTTATGTGTGTTTATTCGAAACTAAG 3' and 5' GTTACCACCATCCAATGCAGACCGTGTATATGAGATAGTTGATTGTATGC 3'. The resulting fragment was fused by PCR to the acl2 gene, isolated as described in example X, using the primers 5' TTAGGCCTGGAACTCCACCGCAC 3' and 5' GTTACCACCATCCAATGCAGACCGTGTATATGAGATAGTTGATTGTATGC 3'. The resulting fusion fragment was ACL-F2

For construction of the fragment ACL-F3, a fragment containing the complete *K. lactis URA3* marker gene upstream of the ADH1 promoter was amplified by PCR from plasmid pWAD2 using the primers 5' CGGTCTGCATTGGATGGTGGTAAC 3' and 5' TGTATATGAGATAGTTGATTGTATGC 3'. The resulting fragment constituted ACL-F3.

For construction of the fragment ACL-F4, a downstream targeting sequence, corresponding to the *POX1* terminator sequence, was amplified by PCR from genomic DNA using the primers 5' GTATCACATTACAATAACAATTCCTTCGAACCCTCTGTTTTGC 3' and 5' TTAGAGCTTCATTCCAACAAGTGCC 3'. The resulting fragment was fused to the acl1 gene, isolated as described in example 32, using the primers 5' GCATACAATCAACTATCTCATATACAATGCCTTCCGCAACTAGCACC 3' and 5' TTAGAGCTTCATTCCAACAAGTGCC 3'.

The yeast strain FS01396 (*MATa ura3-52 trp1 pox1::pTDH3- M.alpina ole1 pADH1-FAS1 pADH1-FAS2*, Example 36) was transformed with the linear fragments ACL-F1, ACL-F2, ACL-F3 and ACL-F4. Transformants were selected and streak-purified on medium lacking uracil and were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype *MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pox1::pTDH3-mole1-pADH1-S.macrospora acl1-pTDH3- S.macrospora acl2* and was named FS01425. Correct assembly of the complete gene targeting substrate and integration at the correct genomic location was verified by colony-PCR.

Example 34

Construction of plasmid pESC-URA-elo-delta-5-AT

The plasmid *pESC-URA-elo-delta-5-AT* contains short versions of the *TDH3* and *ADH1* promoters in replacement of the divergent *GAL10/GAL1* promoter of *pESC-URA-elo-delta-5*. Short versions (fragments corresponding to 674 and 439 bp upstream of the *TDH3* and *ADH1* start codons, respectively) of the *TDH3* and *ADH1* promoters were amplified by PCR using genomic yeast DNA as template and the primer pair 5' AAGCGGCCGCTTTTGTTTGTTTATGTGTGTTTATTCG 3' and 5' AAATGGAAAAAGGGTAGTGAAAAGTTTATCATTATCAATACTGCCATTTC 3' for *pTDH3* amplification and the primer pair 5' TTTCACTACCCTTTTTCCATTTGCCATC 3' and 5' TTCCCGGGTGTATATGAGATAGTTGATTGTATGC 3' for *pADH1* amplification. The two

promoter fragments were then fused by PCR using the primers 5' AAGCGGCCGCTTTTGTTTGTTTATGTGTGTTTATTCG 3' and 5' TTCCCGGGTGTATATGAGATAGTTGATTGTATGC 3'. This resulted in a fragment consisting of the short version of the *TDH3* and *ADH1* promoters in divergent orientation, containing a *NotI* restriction site at the 5' end and an *XmaI* restriction site at the 3' end. Following restriction with *Not*I and *Xma*I, the fragment was introduced into *Not*I/*Xma*I digested *pESC-URA-elo-delta-5*. This resulted in plasmid *pESC-URA-elo-delta-5-AT*, in which the the divergent *GAL10/GAL1* promoter had been replaced by a divergent *TDH3/ADH1* promoter. Absence of PCR-generated mutations in *pESC-URA-elo-delta-5-AT* was verified by sequencing of the *pTDH3/pADH1* promoter sequences.

Example 35

Combining ammonium assimilation modifications with FAS overexpression

Deletion of the *GDH1* gene and overexpression of either the *GDH2* gene or the *GLN1* and *GLT1* genes leads to an altered co-factor dependency of the ammonium assimilation pathway of yeast, and strains carrying these modifications are likely to contain an increased availability of NADPH that can be used for fatty acid synthesis. Deletion of *GDH1* and overexpression of *GDH2* was therefore combined with overexpression of *FAS1* and *FAS2*. Similarly, *GDH1* deletion and overexpression of *GLN1* and *GLT1* was also combined with overexpression of *FAS1* and *FAS2*. This was performed by crossing of strains containing the ammonium assimilation modifications to a strain containing the FAS1 and FAS2 overexpressions. Following sporulation of the resulting diploids, sporulation and dissection of asci, novel haploid strains were identified that carried the desired genetic modifications. The CEN.PK strains used in the present Example carrying the mentioned ammonium assimilation modifications were derived from the strains CEN.MS1-10C T1 and CEN.MS5-3A, which were kind gifts from Margarida Moreira dos Santos, Center for Microbial Biotechnology, Bio-Centrum DTU, Technical University of Denmark. The procedure was as follows:

To combine *GDH1* deletion and *GDH2* overexpression with *FAS1* and *FAS2* overexpression, the yeast strain FS01254 (*MATalpha ura3 gdh1::loxP gdh2::PGKp-GDH2-KanMX3*) was crossed to strain FS01372 (*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2*, Example 26). Diploids were selected on medium lacking uracil and tryptophane and were then transferred on to sporulation medium. Following sporulation, the asci were dissected

into ascospore tetrads. Presence of the modification *gdh2::PGKp-GDH2-KanMX3* in the resulting haploid strains was determined by replica plating to geniticin-containing plates. Presence of the *gdh1::loxP* knockout, the *pADH1-FAS1* overexpression and the *pADH1-FAS2* overexpression were determined by colony PCR using appropriate primers, and remaining genetic features were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP gdh2::PGKp-GDH2-KanMX3* was selected and named FS01398.

To combine *FAS1* and *FAS2* overexpression with *GDH1* deletion, *GLN1* overexpression and *GLT1* overexpression, to consecutive crosses were performed. First, the strain FS01335 (*MATalpha ura3 trp1 gdh1::loxP gln1::PGKp-GLN1-KanMX3 glt1::PGKp-GLT1-KanMX3*) was crossed to FS01372 (*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2*). Diploids were identified by streaking out cells from the cross to single colonies, transferring a number of colonies to a master plate, and testing the ability of the selected colonies to sporulate on sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads. Presence of the modifications *gln1::PGKp-GLN1-KanMX3* and *glt1::PGKp-GLT1-KanMX3* in the resulting haploid strains was determined by replica plating to geniticin-containing plates and by performing colony-PCR using appropriate primers. Presence of the *gdh1::loxP* knockout, the *pADH1-FAS1* overexpression and the *pADH1-FAS2* overexpression were determined by colony PCR, and the mating types were scored using standard methods. From the set of haploid strains derived from the cross, the two strains FS01419 (*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP glt1::PGKp-GLT1-KanMX3*) and FS01420 (*MATalpha ura3 trp1 pADH1-FAS1 gdh1::loxP gln1::PGKp-GLN1-KanMX3*) were selected.

Second, FS01419 and FS01420 were crossed and diploids were selected as described above for the FS01335 x FS01372 cross. Following sporulation, the asci were dissected into ascospore tetrads. Presence of the modifications *gln1::PGKp-GLN1-KanMX3* and *glt1::PGKp-GLT1-KanMX3* in the resulting haploid strains was determined by replica plating to geniticin-containing plates. Presence of the *pADH1-FAS2* overexpression was determined by colony PCR, and the mating types were scored using standard methods. From the set of haploid strains derived from the cross, the strain FS01437 (*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP glt1::PGKp-GLT1-KanMX3 gln1::PGKp-GLN1-KanMX3*) was selected. The presence of the *gdh1::loxP* deletion and the *pADH1-FAS2* overexpression in strain FS01437 was verified by colony PCR.

Example 36

Combining pox1::pTDH3-M. alpina ole1 modification with FAS overexpression and ACC1 overexpression

To combine the genomic integration of *M. alpina ole1* with overexpression of *FAS1* and *FAS2*, FS01368 (*MATalpha ura3 trp1 pox1::pTDH3-M. alpina ole1*, Example 28) was crossed to FS01372 (*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2*, Example 26). Diploids were identified by streaking out cells from the cross to single colonies, transferring a number of colonies to a master plate, and testing the ability of the selected colonies to sporulate on sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads. Presence in the resulting haploid strains of the *pADH1-FAS1* and *pADH1-FAS2* overexpressions and presence of the *pox1:: pTDH3-M. alpina ole1* modification were determined by colony PCR using appropriate primers, and the mating types were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pox1::pTDH3-M. alpina ole1* was selected and named FS01396. Furthermore, a strain with the genotype *MATalpha ura3 trp1 pADH1-FAS1 pox1::pTDH3-M. alpina ole1* was selected and named FS01408.

To further combine genomic integration of *M. alpina ole1*, overexpression of *FAS1*, and overexpression of *FAS2* with *ACC1* overexpression, FS01408 (*MATalpha ura3 trp1 pADH1-FAS1 pox1::pTDH3-M. alpina ole1*, derived as described above) was crossed to FS01392 (*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pTPI1-ACC1*, Example 27). Diploids were selected as described above for the FS01368 x FS01372 cross and were transferred to sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads. Presence in the resulting haploid strains of the *pADH1-FAS2* and *pTPI1-ACC1* overexpressions and presence of the *pox1:: pTDH3-M. alpina ole1* modification were determined by colony PCR using appropriate primers, and the mating types were scored using standard methods. From the set of haploid strains derived from the cross, a strain with the genotype *MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pTPI1-ACC1 pox1::pTDH3-M. alpina ole1* was selected and named FS01423.

Example 37

Expression of pathway to arachidonic acid in genetically modified yeast

To express the pathway to arachidonic acid in the modified strain backgrounds described in the previous Examples, the engineered strains were co-transformed with the plasmids pESC-TRP-delta-12 delta-6 and pESC-URA-elo-delta-5. An overview of the strains resulting from the transformations is shown in Table 4.

Table 4. Summary of arachidonic acid producing strains, their genotypes and parent strains.

Strain	Genotype	Parent
FS01373	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01372
FS01413	*MATa ura3 trp1 pADH1-FAS1* p*ADH1-FAS2 pTPI1-ACC1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01392
FS01369	*MAT alpha ura3 trp1 pox1::pTDH3-M.alpina ole1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01368
FS01371	*MATa ura3 trp1 pTDH3-DGA1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01370
FS01417	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pox1::M. alpina ole1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01396
FS01414	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-YDR531W* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01393
FS01415	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-SLC1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01394
FS01416	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pTPI1-GAT1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01395
FS01418	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP gdh2::PGKp-GDH2-KanMX3* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01398
FS01429	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pTPI1-ACC1 pox1::M. alpina ole1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01423
FS01430	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 pox1::pTDH3-mole1-pADH1-S. macrospora acl1-pTDH3- S. macrospora acl2* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01425

FS01431	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pADH1-YBR159W* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01427
FS01439	*MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 gdh1::loxP glt1::PGKp-GLT1-KanMX3 gln1::PGKp-GLN1-KanMX3* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01437
FS01442	*MATa ura3 trp1* p*ADH1-FAS1* p*ADH1-FAS2 pADH1-TSC13* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01440

Example 38

Fatty acid compositions of arachidonic acid producing yeast strains in shake flasks

The reference strain FS01324 (*MATa ura3 trp1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]) and the metabolically engineered strains FS01373, FS01413, FS01369, FS01371, FS01417, FS01414, FS01415, FS01416, FS01418, FS01429, FS01430, FS01431, FS01439 and FS01442 (Example 37) were cultured in shake flasks at 17°C as described in example 9. Following depletion of carbon source, lipids were extracted and the fatty acid compositions of the strains were analyzed as described in Example 45. The results of the analysis are shown in Table 5. All samples were analyzed on a SP-X column, which gives full separation of the peaks. For comparison, the results shown in Example 12 (Table 2) were obtained using a JW-1701 column, which does not give complete separation of the peaks, and thereby results in overestimation of the area percentage of the smaller peaks.

The introduction of *M. alpina ole1*, encoding a fungal delta-9 desaturase with higher specificity towards 18:0 than 16:0, resulted in a decreased level of 16:1 and a corresponding increase in 18:1 and PUFAs (FS01369, Table 5a). The content of arachidonic acid was approximately doubled in FS01369 compared to the reference strain FS01324 (0.63% compared to 0.28% of total fatty acid).

Overexpression of *FAS1* and *FAS2* (strain FS01373) or *FAS1*, *FAS2* and *ACC1* (strain FS01413) resulted in a slight increase in ARA content compared to the reference strain FS01324 (Table 5a). In the strain FS01417, *FAS1* and *FAS2* overexpression was combined with expression of *M. alpina ole1*. The fatty acid composition of this strain was

similar to the composition of strain FS01369 (carrying the M. alpina ole1 as the sole genomic modification).

Overexpression of *YDR531W*, putatively encoding pantothenate kinase, in strain FS01414 did not lead to significant alterations of the fatty acid composition compared to the reference strain. *GAT1*, *SLC1* and *DGA1*, all encoding acyltransferases involved in TAG synthesis, were overexpressed separately (strain FS01416, FS01415 and FS01371, respectively). For all of these three genes, overexpression resulted in decreased levels of arachidonic acid (Table 5a, 5b). In addition, for overexpression of SLC1 and GAT1, the 16:1 content was increased by 10% of total FA.

The strain FS01418, carrying a deletion of *GDH1* and overexpressing *GDH2* in addition to *FAS1* and *FAS2*, contained decreased amounts of arachidonic acid compared to FS01373 (overexpressing *FAS1* and *FAS2*) (Table 5b). The strain FS01439, carrying a deletion of *GDH1* and overexpressing *GLN1* and *GLT1* in addition to overexpression of *FAS1* and *FAS2*, contained 0.44% arachidonic acid compared to 0.39% in FS01373.

The strain FS01430 carries the two genes encoding subunits of ATP:citrate lyase from the oleagineous fungus *Sordaria macrospora* under the control of strong constitutive yeast promoters. In addition, it overexpresses *FAS1*, *FAS2* and *M. alpina ole1*. Typical for the strains expressing *M. alpina ole1*, the content of 16:1 was decreased in this strain compared to the reference strain FS01324. FS01430 had a higher 18:1 and 18:2 content, and a slightly increased ARA content compared to its parent strain FS01417 (overexpressing *FAS1*, *FAS2* and *M. alpina ole1*).

In the strain FS01431, the gene *YBR159W*, encoding β-ketoacyl-CoA reductase, was overexpressed in a *FAS1*/*FAS2* overexpressing background. FS01431 did not show an increased ARA content compared to the strain FS01373 (overexpressing FAS1 and FAS2), but instead contained increased levels of 16:1 (Table 5b). However, the elongation of 18:3 to 20:3 was improved from 19% conversion in FS01373 to 25% conversion in FS01431, calculated as 100x (20:3 + 20:4)/(18:3 + 20:3 + 20:4).

Table 5a. Fatty acid composition (% of total fatty acid) of metabolically engineered strains expressing the pathway to arachidonic acid, analyzed in shake flasks.

Fatty acid	FS01324	FS01369	FS01373	FS01413	FS01417	FS01414	FS01415	FS01416
12:0	1,07	1,63	1,25	1,44	7,30	1,63	1,17	1,43
16:0	19,03	19,87	16,53	15,50	21,21	15,16	14,28	13,00
Δ9-16:1	40,11	28,33	39,69	39,49	27,05	39,65	50,03	51,21
16:2	3,50	2,83	4,78	3,28	1,86	6,35	5,35	5,65
18:0 stearic acid	8,81	4,55	8,37	4,86	4,62	5,14	4,12	3,82
16:3	0,72	0,72	1,30	0,81	0,48	2,20	1,56	1,65
Δ9-18:1 oleic acid	12,31	18,19	12,10	11,64	17,74	12,26	9,71	9,23
Δ9, Δ12-18:2 linoleic acid	8,00	10,81	9,74	7,07	8,24	8,50	7,69	7,13
Δ6, Δ9, Δ12-18:3 gamma-linolenic acid	2,97	5,52	3,77	2,66	6,35	4,07	3,18	3,93
Δ8, Δ11, Δ14-20:3 di-homo-gamma-linolenic acid	0,34	1,55	0,51	0,28	1,53	0,47	0,27	0,23
Δ5, Δ8, Δ11, Δ14-20:4 arachidonic acid	0,28	0,63	0,39	0,41	0,35	0,35	0,25	0,15
Others	2,86	5,37	1,56	12,56	3,27	4,24	2,39	2,57
Sum	100	100	100	100	100	100	100	100

Table 5b. Fatty acid composition (% of total fatty acid) of metabolically engineered strains expressing the pathway to arachidonic acid, analyzed in shake flasks.

Fatty acid	FS01371	FS01418	FS01430	FS01431	FS01442	FS01439	FS01429
12:0	1,51	0,98	0,94	0,09	0,57	1,02	1,40
16:0	17,20	15,14	18,12	11,47	19,76	13,27	21,47
Δ9-16:1	41,53	41,73	27,58	50,28	33,84	33,62	26,42
16:2	3,79	4,44	1,48	2,96	7,51	7,84	2,44
18:0	4,98	6,79	2,93	4,61	7,56	7,24	4,41
16:3	1,01	0,61	0,24	0,67	0,88	1,06	0,17
Δ9-18:1 oleic acid	14,56	11,98	28,07	17,05	8,76	11,40	23,16
Δ9, Δ12-18:2 linoleic acid	7,58	8,49	14,02	7,93	11,74	16,87	15,43
Δ6, Δ9, Δ12-18:3 gamma-linolenic acid	3,65	2,03	3,80	2,74	2,70	4,08	1,77
Δ8, Δ11, Δ14-20:3 di-homo-gamma-linolenic acid	0,39	0,19	0,81	0,49	2,02	0,63	0,72
Δ5, Δ8, Δ11, Δ14-20:4 arachidonic acid	0,14	0,17	0,50	0,43	0,75	0,44	0,25
Others	3,67	7,45	1,52	1,27	3,92	2,54	2,36
Sum	100	100	100	100	100	100	100

In the strain FS01442, the gene *TSC13*, encoding trans-2-enoyl-CoA reductase, was overexpressed in a *FAS1*/*FAS2* overexpressing background. This resulted in a marked increase in arachidonic acid content, from 0.39% of total fatty acid in FS01373 (overexpressing *FAS1* and *FAS2*) to 0,75% of total fatty acid in FS01442 (overexpressing *FAS1*, *FAS2* and *TSC13*). The increase in arachidonic acid content was due to increased elongation efficiency, from 19% conversion in FS01373 to 51% conversion in FS01431, calculated as 100x (20:3 + 20:4)/(18:3 + 20:3 + 20:4).

Example 39

Lipid yields of arachidonic acid producing yeast strains in shake flasks

The reference strain FS01324 (*MATa ura3 trp1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]) and the metabolically engineered strains FS01373, FS01413, FS01369, FS01371, FS01417, FS01414, FS01415, FS01416, FS01418, FS01429, FS01430, FS01431 and FS01439 (Example 37) were cultured at 17°C as described in Example 9. Lipids were extracted and quantified as described in Example 44. The lipid yields, biomass yields and arachidonic acid yields of the modified strains are summarized in Table 6.

Table 6. Lipid yield, biomass yield and arachidonic acid yield in arachidonic acid producing strains, analyzed in shake flasks. The yield of arachidonic acid on carbon-source was calculated under the assumption that fatty acids constitute 70% (w/w) of lipids. Lipids were extracted shortly after depletion of the carbon source for all strains except the strains marked by an asterisk (*); for these strains lipids were extracted at mid-exponential phase.

Strain	Lipid yield (% of dw)	arachidonic acid (% of FA)	Biomass yield (g dw/g hexose)	arachidonic acid yield (mg/g hexose)
FS01324	12.8 ± 1.9	0.31	0.36	0.10
FS01369*	9.9 ± 1.1	0,63	n.a.	n.a.
FS01373	11.4 ± 0.3	0.47	0.34	0.13
FS01413	16.8 ± 0.8	0.41	0.33	0.16
FS01417	20.1 ± 0.14	0.35	0.28	0.14
FS01414	17.0 ± 0.8	0.35	0.33	0.14
FS01415	17.5 ± 0.3	0.25	0.33	0.10
FS01416	17.8 ± 0.3	0.15	0.35	0.07
FS01418	17.0 ± 1.5	0.17	0.26	0.05
FS01430	16.8 ± 0.6	0.50	0.30	0.18
FS01431	12.8 ± 0.2	0.43	0.29	0.11
FS01371*	8.9 ± 1.2	0,14	n.a.	n.a.
FS01439*	16.5 ± 3.5	0,44	n.a.	n.a.
FS01429*	10.3 ± 1.1	0,25	n.a.	n.a.
FS01442*	8.6 ± 1.2	0,75	n.a.	n.a.

According to this analysis, overexpression of *FAS1* and *FAS2* alone (strain FS01373) did not result in increased lipid yield compared to the reference strain FS01324. However,

FAS1/FAS2 overexpression combined with other modifications (overexpression of either *ACC1*, *YDR531W*, *SLC1*, *GAT1* or *S. macrospora acl1* and *acl2*, *POX1* deletion and *M.alpina ole1* overexpression, *GDH1* deletion combined with *GDH2* overexpression, *GDH1* deletion combined with *GLN1* and *GLT1* overexpression) did result in increased lipid yield compared to the reference strain. The most successful strain in terms of arachidonic acid yield on carbon source was FS01430 (overexpressing *FAS1*, *FAS2*, *S. macrospora* acl1 and acl2, and *M. alpina ole1*). This strain produced 0.18 mg arachidonic acid/g hexose, compared to 0.10 mg/g hexose in the reference strain FS01324.

Example 40

Chemostat fermentations

Continuous cultivations were performed in Braun Biostat B fermenters (Braun Biotech International). Cells from a 48 h shake flask culture in defined minimal medium (Verduyn et al,. 1990) were used for inoculation of 1.0 l medium to an OD_{600} of 0.2 as measured by using a Hitachi U-1100 spectrophotometer (Tokyo, Japan). The fermentations were carried out at 17 °C and at pH 5.0, controlled by 2M KOH. Foaming was avoided by the addition of 100 µl Antifoam 204 (Sigma-Aldrich, St Louis, Missouri) per liter medium. Aerobic conditions were obtained by sparging the fermentor with sterile air at a flow rate of 1.5-2.5 l/min to ensure that the dissolved oxygen concentration was above 60%. The stirring speed was kept at 800 rpm and the carbon dioxide content of outflowing gas was measured with a Brüel and Kjær acoustic gas analyzer (Brüel & Kjær, Denmark). Following depletion of the carbon source, level controlled continuous fermentation mode at a dilution rate of 0.05 h^{-1} was applied.

Example 41

Growth media used in chemostat fermentations

Carbon-limited media with glucose or ethanol as carbon source contained: 12.5 g/l glucose or 10 g/l ethanol, 5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 1ml/l vitamin solution and 1 ml/l trace metal solution. Nitrogen-limited media with glucose or ethanol as carbon source contained: 40 g/l glucose or 30 g/l ethanol, 2 g/l $(NH_4)_2SO_4$, 4 g/l K_2SO_4, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4 * 7H_2O$, 1ml/l vitamin solution and 1 ml/l trace metal solution. The glucose/galactose carbon-limited minimal medium contained: 2.5 g/l glucose, 10 g/l galactose, 5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 1ml/l vitamin solution and 1 ml/l trace metal solution. For all media described above, the vitamin solution contained: 50 mg/L biotin, 1 g/L calcium panthotenate, 1 g/L nicotinic acid, 25 g/L myo-inositol, 1 g/L thiamine HCl, 1 g/L pyridoxal HCl and 0.2 g/L para-aminobenzoic acid, while the trace

metal solution contained: 15 g/L EDTA, 4.5 g/L $ZnSO_4 \cdot 7H_2O$, 1 g/L $MnCl_2 \cdot 2H_2O$, 0.3 g/L $CoCl_2 \cdot 6H_2O$, 0.4 g/L $Na_2MoO_4 \cdot 2H_2O$, 4.5 g/L $CaCl_2 \cdot 2H_2O$, 3 g/L $FeSO_4 \cdot 7H_2O$, 1 g/L H_3BO_3 and 0.1 g/L KI. The improved myo-inositol deficient medium contained no myo-inositol but was otherwise identical to the glucose/galactose carbon-limited minimal medium.

Example 42

HPLC analysis

Glucose, galactose, ethanol, glycerol, acetate, succinate, and pyruvate concentrations in the culture broth were determined by column liquid chromatography (CLC) using a Dionex Summit CLC system (Dionex, Sunnyvale, CA) after removing the cells from the culture broth via centrifugation. An Aminex HPX-87H column (BioRad, Hercules, CA) was used at 60°C with a Waters 410 Differential refractive index detector (Millipore, Milford, MA) and a Waters 486 Tuneable Absorbance Detector (UV detector) set at 210 nm. The two detectors were connected in series. As mobile phase 5 mM H_2SO_4 was used at a flow rate of 0.6 ml/min.

Example 43

Biomass dry weight determination

The cell dry weight was determined by filtering a known volume of the culture broth through a pre-weighed 0.45 µm Supor membrane (Pall Corporation, Ann Arbor, MI) filter. After washing with 1 volume of distilled water and drying in microwave oven for 15 minutes at 150 W, the filter was weighed again.

Example 44

Total lipid analysis

For analysis of total lipid yield, the biomass was separated through centrifugation for 5 minutes at 5000 rpm. The biomass was re-dissolved in 10 ml distilled water and the resulting cell suspension was broken using the glass bead method to generate cell extract. The cell extract was prepared by addition of 1 ml glass beads with a particle size of 250-500 µm (Sigma-Aldrich, St Louis, Missouri) to 1 ml cell suspension in a micro tube with screw cap (Sarstedt, Germany). For each cell suspension 6 tubes were processed. The tubes were shaken at level 4 for 20 seconds in a FastPrep FP120 instrument (Qbiogene, France). This was done in total 6 rounds for each tube with a 5 minutes intervening cooling of the tubes on ice after 3 rounds. The cell extracts were combined in 2 ml eppendorf

tubes by transferring 600 µl cell extract to generate 3 eppendorf tubes each containing 1.2 ml glass bead free cell extract. 1 ml of the cell extract was transferred into a glass tube with screw cap containing 20 ml chloroform/methanol 2:1. The tube was sparged with nitrogen then closed immediately and placed on a rotary mixer and the total lipid extraction was performed over night. This was done in triplicates. The extract was then filtered through a Whatman filter (Whatman International, England) and the collected solvent was washed with 4 ml NaCl and finally dried over nitrogen in pre-weighed 10 ml glass tubes. The tubes with dry lipid fraction were weighed and the lipid yield was determined by calculating the lipid dry weight divided by the dry weight of the biomass in 1 ml of the initial cell suspension.

Example 45

Transesterification of lipids and GC-MS analysis

Dry lipid was generated as for the determination of total lipid (Example 44) and was dissolved in 1 ml toluene and 2 ml 1 % sulphuric acid in methanol was added. The tube was closed after mixing and sparging with nitrogen and left at 50°C over night for transesterification of the lipids. The sample was then washed with 5 ml 5% NaCl solution. Methyl esters were subsequently extracted twice by adding 5 ml hexane, vortexing the sample and collecting the organic upper phase. The organic phase was washed with 4 ml 2% sodium carbonate and the organic phase was collected again. Trace of water phase was removed by adding anhydrous sodium sulphate and filtering the sample through a Whatman filter paper (Whatman International, England) to remove the sodium sulphate. The hexane phase was then dried under a stream of nitrogen. When dry, the sample was redissolved in 0.5 ml of hexane containing 0.01% butylated hydroxytoluene (BHT) (Sigma-Aldrich, St Louis, Missouri) for protection of double bonds was added and the sample was analyzed for methyl esters. The analysis was performed using a gas chromatograph coupled to mass selective detector (GC/MS). The GC/MS system was a Hewlett Packard HP G1723A with a gas chromatograph-quadruple mass selective detector (EI) operated at 70 eV. The column used was Supelco SP™-2380. The MS was operated in SCAN Mode. The oven temperature was initially 170°C and in the following risen to 220°C at 4°C/min. The final temperature was held for 15 min. The flow through the column was 0.6 ml He/min. Injection volumes were 1 - 5 µl. The injector was driven at split of 100:1 splitless for all analyses. The temperature of the inlet was 300°C, the interface temperature 230 °C, and the quadropule temperature 105°C. Detected fatty acid methyl esters were confirmed with the 1998 NIST Mass Spectral Database, and retention times were confirmed with standard fatty acid methyl esters.

Example 46

Analysis of arachidonic acid producing yeast strains in continuous fermentation

The reference strain FS01324 (*MATa ura3 trp1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]) and the metabolically engineered strains FS01373 and FS01413 (Example 37) were grown in chemostat cultivations as described in Example 40. The medium was glucose/galactose carbon-limited minimal medium as described in Example 41. At steady state, samples were taken for HPLC analysis, analysis of biomass dry-weight, lipid yield and fatty acid composition as described in the above Examples. A summary of the analysis is shown in Table 7.

Table 7. Arachidonic acid content (% of total fatty acid), lipid yield (% of biomass dry-weight), biomass yield (g dry-weight/g hexose), arachidonic acid yield on biomass (mg arachidonic acid/ g biomass dry-weight) and arachidonic acid yield on carbon source (mg arachidonic acid/ g hexose) of arachidonic acid producing yeast strains analyzed in continuous fermentation with standard carbon-limited minimal medium. The yield of arachidonic acid on carbon-source was calculated under the assumption that fatty acids constitute 70% (w/w) of lipids.

Strain	**20:4**	**Lipid yield**	**Biomass yield**	**20:4 yield on biomass**	**20:4 yield on c- source**
	(% of FA)	**(% of dw)**	**(g /g)**	**(mg/g)**	**(mg/ g)**
FS01324	1.1	15.0 ± 0.6	0.43	1.17	0.50
FS01373	1.0	15.3 ± 0.5	0.42	1.03	0.44
FS01413	0.8	12.1 ± 2.9	0.56	0.64	0.36

All strains had a higher content of arachidonic acid in chemostat cultivations than previously shown in shake flask cultures. However, the genetically modified strains FS01373 and FS01413 did not have improved lipid yields relative to the reference strain FS01324 in carbon-limited chemostat cultures.

Example 47

Medium optimization for increased arachidonic acid yield

The wild-type strain CEN.PK 113-7D, the reference strain FS01324 (*MATa ura3 trp1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]), and the metabolically engineered strains FS01373, FS01413, FS01417 and FS01430 (Example 37) were grown in chemostat cultivations as described in Example 40. The medium used in these experiments was an

improved carbon-limited minimal medium, which contained no myo-inositol (Example 41). At steady state, samples were taken for HPLC analysis, analysis of biomass dry-weight, lipid yield and fatty acid composition as described in the above Examples. A summary of the analysis is shown in Table 8.

Table 8. Arachidonic acid content (% of total fatty acid), lipid yield (% of biomass dry-weight), biomass yield (g dry-weight/g hexose), arachidonic acid yield on biomass (mg arachidonic acid/ g biomass dry-weight) and arachidonic acid yield on carbon source (mg arachidonic acid/ g hexose) of arachidonic acid producing yeast strains analyzed in continuous fermentation with myoinositol-deficient, carbon-limited minimal medium. The yield of arachidonic acid on carbon-source was calculated under the assumption that fatty acids constitute 70% (w/w) of lipids.

Strain	20:4	Lipid yield	Biomass yield	20:4 yield on biomass	20:4 yield on c- source
	(% of FA)	(% of dw)	(g /g)	(mg/g)	(mg/ g)
CEN.PK	0	8.4 ± 2.5	0.67	0	0
FS01324	0.8	10.4 ±1.0	0.75	0.58	0.43
FS01373	1.2	11.7 ± 1.0	0.67	1.01	0.68
FS01413	1.5	10.4 ± 0.2	0.68	1.09	0.74
FS01430	2.7	8.6 ± 0.7	0.58	1.62	0.72
FS01417	3.5	8.0 ± 0.8	0.45	1.93	1.12

In myo-inositol deficient, carbon-limited medium, the metabolically engineered strains FS01324, FS01373, FS01413, FS01430 and FS01417 all contained higher levels of arachidonic acid than the reference strain FS01324. In particular, the strain FS01417 contained 3.5 % arachidonic acid of total fatty acid, compared to 0.8 % in the reference strain FS01324. An overview of the performance of the genetically engineered strains is shown in Figure 18.

Example 48

Medium optimization for increased total lipid yield

In contrast to the results of shake flask experiments (Example 39), the modified strains did not have increased lipid yields relative to the reference strain in the chemostat cultivations of Examples 46 and 47. This is probable to be due to that the experiments were carried out under carbon-limitation, which is not optimal for lipid accumulation. It is

therefore likely that the metabolically engineered strains will show increased lipid yields relative to the reference strain when analyzed under conditions that promote lipid accumulation. To optimize the chemostat conditions for lipid accumulation, the wild-type strain CEN.PK 113-7D was grown in chemostat as described in Example 40 in different medium compositions. The media used in these experiments were either i) carbon-limited with glucose as carbon source, ii) nitrogen-limited with glucose as carbon source, iii) carbon-limited with ethanol as carbon-source or iiii) nitrogen-limited with ethanol as carbon source (Example 41). At steady state, samples were taken for HPLC analysis, analysis of biomass dry-weight and lipid yield as described in the above Examples. A summary of the analysis is shown in Table 9.

Table 9. Biomass yield and lipid yield of the wild-type strain CEN.PK 113-7D grown in chemostat with i) carbon-limited medium with glucose as carbon source, ii) nitrogen-limited medium with glucose as carbon source, iii) carbon-limited medium with ethanol as carbon-source or iiii) nitrogen-limited medium with ethanol as carbon source.

Medium	**Lipid yield (% of dw)**
i) C-lim, glc	7
ii) N-lim, glc	14
iii) C-lim, EtOH	10
iiii) N-lim, EtOH	10

The analysis showed that the lipid yield in chemostat cultivation with *S. cerevisiae* can be approximately doubled by using a nitrogen-limited, rather than glucose-limited, medium.

Example 49

Analysis of arachidonic acid producing yeast strains in continuous fermentation with optimized growth medium

The reference strain FS01324 (*MATa ura3 trp1* [pESC -TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]) and the engineered strains FS01373, FS01413, FS01369, FS01371, FS01417, FS01414, FS01415, FS01416, FS01418, FS01429, FS01430, FS01431, FS01439 and FS01442 (Example 37) are grown in continuous cultivations using nitrogen-limited, myo-inositol deficient medium. At steady state, samples are taken for HPLC analysis, analysis of biomass dry-weight, lipid yield and fatty acid composition as described in the above Examples. The use of nitrogen-limited medium is anticipated to lead to higher lipid yields and therefore increased arachidonic acid yields. In addition, the strains carrying genetic

modifications aimed at improving lipid yield are likely to have even higher lipid yields than the reference strain in nitrogen-limited medium.

Example 50

Construction of p300, a single yeast expression vector containing genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase

To reduce the number of plasmids needed for expression of PUFA pathways, the four genes required for arachidonic acid production (*M. alpina* genes encoding -12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase) were placed on a single plasmid, p300. The strategy used for construction of p300 is shown in Figure 19. First, a second PacI restriction site was introduced into pESC-TRP-delta-12 delta-6 at the NheI restriction site. For this purpose, the palindromic synthetic oligonucleotide 5´ CTAGCAATTCCTTAATTAAGGAATTG 3´ was used. This oligonucleotide anneals to itself, resulting in a double-stranded DNA fragment containing a PacI restriction site and overhangs at both ends that match the NheI restriction site. The fragment was cloned into the NheI restriction site of pESC-TRP-delta-12 delta-6, resulting in the plasmid pESC-TRP-delta-12 delta-6-PacI. Next, a fragment containing the ADH1 and CYC1 terminator sequences in back-to-back orientation was introduced into pESC-TRP-delta-12 delta-6-PacI. To construct the ADH1-CYC1-terminator fragment, the ADH1 terminator was amplified by PCR using the primers 5′ TGTTCTCGAGAAGGTGTTGAGCGACCTCATGCTATACCTGAGAAAG 3′ and 5′ CCATCGATGGCGAATTTCTTATGATTTATGATTTTTA 3′ and the CYC1 terminator was amplified by PCR using the primers 5′ GAAGATCTTCCCGCTCTAACCGAAAAGGAAGG 3′ and 5′ AACACCTTCTCGAGAACACTTCGAGCGTCCCAAAACC 3′, using pESC-URA as template for both reactions. Following purification of the ADH1 and CYC1 terminator fragments, they were fused by PCR using the primers 5′ GAAGATCTTCCCGCTCTAACCGAAAAGGAAGG 3′ and 5′ CCATCGATGGCGAATTTCTTATGATTTATGATTTTTA 3′. The ClaI and BglII restriction sites included in these primers allowed introduction of the ADH1-CYC1-terminator fragment into ClaI/BglII digested pESC-TRP-delta-12 delta-6-PacI, resulting in the plasmid pESC-TRP-delta-12 delta-6-PacI-T. Absence of mutations in the terminator sequences was verified by sequencing of pESC-TRP-delta-12 delta-6-PacI-T. To construct the plasmid p300, pESC-TRP-delta-12 delta-6-PacI-T was digested with *Pac*I, resulting in release of an insert containing the *M. alpina* gene encoding delta-12 desaturase, the divergent GAL1/GAL10 promoter, the *M. alpina* gene encoding delta-6 desaturase, the *ADH1* terminator and the *CYC* terminator (Figure 19). The insert was purified and introduced into *Pac*I digested pESC-URA-elo-delta-5, resulting in plasmid p300. The correct orientation of the pESC-TRP-delta-12 delta-6-PacI-T-derived insert in p300 was verified by restriction analysis.

Example 51

Cloning of an omega-3 desaturase from A. thaliana into a yeast expression vector

The *A. thaliana FAD3* gene (SEQ ID NO 32), encoding an omega-3 desaturase, was amplified from an *A. thaliana* cDNA preparation (Plant normal Tissue First strand cDNA/Arabidopsis, Gentaur Molecular Products) using the primers 5' GGTCTCGAGCCACCATGGTTGTTGCTATGGACCAAC 3' and 5' GGGGTACCATTAATTGATTTTAGATTTGTCAGAAGCGTAA 3'. These primers introduce XhoI and KpNI restriction sites at the 5' and 3' end of the gene, respectively. The *A. thaliana FAD3* gene was introduced into XhoI/KpNI digested pESC-TRP, resulting in the plasmid pESC-TRP-Aro3. Absence of mutations in *A. thaliana FAD3* was verified by sequencing of pESC-TRP-Aro3.

Example 52

Cloning of omega-3 desaturase from Saccharomyces kluyveri into a yeast expression vector

The *S. kluyveri FAD3* gene (SEQ ID NO 87), encoding an omega-3 desaturase, was amplified from a preparation of genomic DNA from *S. kluyveri* Y159 using the primers 5' GGTCTCGAGCCACCATGTCTATTGAAACAGTCGG 3' and 5' GGCCGCGGATCATTGACTGGAACCATCTT 3'. These primers introduce XhoI and SacII restriction sites at the 5' and 3' end of the gene, respectively. The *S. kluveri FAD3* gene was introduced into XhoI/SacII digested pESC-TRP, resulting in the plasmid pESC-TRP-SK33. Absence of mutations in *S. kluveri FAD3* was verified by sequencing of pESC-TRP-SK33.

Example 53

Expression of omega-3 desaturase from S. kluyveri and evaluation of p300

Yeast strain FS01267 (MATa trp1 ura3) was co-transformed with plasmids p300 and pESC-TRP-SK33. Transformants were selected on medium lacking uracil and tryptophane and were streak purified on the same medium. The transformed strain was named FS01432. FS01432 was grown in a shake flask as described in example 9 and the fatty acid composition was analyzed as described in Example 45. The fatty acid compositions of FS01432, the reference strain FS01324 and *S. kluyveri* Y159 are shown in Table 10.

The results of the analysis showed that strain FS01432, expressing the pathway to arachidonic acid and the omega-3 desaturase from *S. kluyveri*, contained alpha-linoleic acid and small amounts of stearidonic acid. The alpha-linolenic acid content in this strain was higher than the gamma-linolenic acid content, indicating that the omega-3 desaturase from *S. kluyveri* desaturates linoleic acid more efficiently than the delta-6 desaturase from *M. alpina*.

Table 10. Fatty acid composition (% of total fatty acid) of strain FS01324 (MATa ura3 trp1 [pESC-TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]), FS01432 (MATa ura3 trp1 [p300] [pESC-TRP-SK33]) and *S. kluyveri* Y159.

Fatty acid	**FS01324**	**FS01432**	***S. kluyveri* Y159**
12:0 dodecanoic acid	1,1	1,2	
16:0 palmitic acid	19,0	15,3	23,9
Δ9-16:1 palmitoleic acid	40,1	46,1	20,5
16:2	3,5	1,7	
18:0 stearic acid	8,8	4,8	4,8
16:3	0,7		
Δ9-18:1 oleic acid	12,3	20,9	31,7
Δ9, Δ12-18:2 linoleic acid	8,0	3,4	14,7
Δ6, Δ9, Δ12-18:3 gamma-linolenic acid	3,0	0,4	
Δ9, Δ12, Δ15-18:3 alpha-linolenic acid		2,2	4,4
Δ6, Δ9, Δ12, Δ15-18:4 stearidonic acid		0,1	
Δ8, Δ11, Δ14-20:3 di-homo-gamma-linolenic acid	0,3	0,03	
Δ5, Δ8, Δ11, Δ14-20:4 arachidonic acid	0,3		
Others	2,8	3,9	

Sum	100	100	100

Comparing the fatty acid composition of FS01324 (expressing the pathway to ARA from two plasmids) and FS01432 (expressing the pathway to ARA from a single plasmid), it can be observed that the conversion of the initial substrate oleic acid into linoleic acid and intermediates downstream of linoleic acid is less efficient in FS01432 than FS01324. Thus, FS01432 contains only 6.1% fatty acids with two or more double bonds compared to 11.6% in FS01324. This decrease is also reflected in the higher content of oleic acid in FS01432 compared to FS01324. In conclusion, it appears that expression from plasmid p300 is less efficient than expression from the original plasmids pESC-TRP-delta-12 delta-6 and pESC-URA-elo-delta-5.

Example 54

Construction of the vector pESC-LEU-SK33

To construct vector pESC-LEU-SK33, *S. kluyveri FAD3* was released from pESC-TRP-SK33 (Example 52) by digestion with ApaI and NheI and was introduced into ApaI/NheI digested pESC-LEU, resulting in the plasmid pESC-LEU-SK33 (Figure 20A). Correct insertion of *S. kluyveri FAD3* was verified by restriction analysis.

Example 55

Construction of the vector pESC-LEU-Ssc2

To construct vector pESC-LEU-Ssc2, mouse *Ssc2* was released from pESC-TRP-delta-5elo (Example 15) by digestion with BglII and PacI and was introduced into BglII/PacI digested pESC-LEU, resulting in the plasmid pESC-LEU-Ssc2 (Figure 20B). Correct insertion of mouse *Ssc2* was verified by restriction analysis.

Example 56

Construction of the vector pESC-LEU-Ssc2-SK33

To construct vector pESC-LEU-Ssc2-SK33, *S. kluyveri FAD3* was released from pESC-TRP-SK33 (Example 52) by digestion with ApaI and NheI and was introduced into ApaI/NheI digested pESC-LEU-Ssc2 (Example 55), resulting in the plasmid pESC-LEU-Ssc2-SK33 (Figure 20C). Correct insertion of *S. kluyveri FAD3* was verified by restriction analysis.

Example 57

Introducing leu2 mutation in pox1::pTDH3-M. alpina ole1 background

To enable expression of a PUFA pathway from three plasmids, carrying *URA3*, *TRP1* and *LEU2* markers, respectively, in a strain carrying the *M. alpina ole1* integrated into its genome, a l*eu2* mutation was introduced into this strain background. For this purpose, FS01368 (*MATalpha ura3 trp1 pox1::pTDH3-M.alpina ole1)* was crossed to FS01277 (*MATa ura3 leu2 trp1*). Diploids were identified by streaking out cells from the cross to single colonies, transferring a number of colonies to a master plate, and testing the ability of the selected colonies to sporulate on sporulation medium. Following sporulation, the asci were dissected into ascospore tetrads and the genotypes were scored. Presence of the *pox1:: pTDH3-M. alpina ole1* modification was determined by colony PCR using appropriate primers. From the set of haploid strains derived from the cross, a strain with the genotype *MATalpha ura3 trp1 leu2 pox1::pTDH3-M. alpina ole1* was selected and named FS01444.

Example 58

Expression of the pathways to eicosapentaenoic acid, docosatetraenoic acid and docosapentaenoic acid in S. cerevisiae

To express the pathways to eicosapentaenoic acid, docosatetraenoic acid and docosapentaenoic acid in *S. cerevisiae*, the plasmids constructed in Examples 54-56 were introduced into yeast together with the two plasmids pESC-TRP-delta-12 delta-6 and pESC-URA-elo-delta-5, resulting in the strains FS01446, FS01447 and FS01448 (Table 11).

Table 11. Genotypes of the strains FS01369, FS01446, FS01447 and FS01448

Strain	Genotype	Parent
FS01369	*MAT alpha ura3 trp1 pox1::pTDH3-M.alpina ole1* [pESC - TRP-delta-12 delta-6] [pESC-URA-elo-delta-5]	FS01368
FS01446	*MATalpha ura3 trp1 leu2 pox1::pTDH3-M. alpina ole1* [pESC-TRP-delta-12 delta-6] [pESC-URA-elo-delta-5] [pESC-LEU-SK33]	FS01444
FS01447	*MATalpha ura3 trp1 leu2 pox1::pTDH3-M. alpina ole1* [pESC-TRP-delta-12 delta-6] [pESC-URA-elo-delta-5] [pESC-LEU-Ssc2]	FS01444
FS01448	*MATalpha ura3 trp1 leu2 pox1::pTDH3-M. alpina ole1* [pESC-TRP-delta-12 delta-6] [pESC-URA-elo-delta-5] [pESC-LEU-Ssc2-SK33]	FS01444

The strains FS01446, FS01447 and FS01448 and FS01369 were first cultured in shake flasks with 150 rpm shaking at 17°C in defined minimal medium containing 5 g/l glucose and 20 g/l galactose. Following depletion of glucose and while the cells were in exponential growth on galactose, 1 ml of each culture was transferred to a new shake flask containing 100 ml minimal medium with 20 g/l galactose as the sole carbon source. The cells were then cultured at 17°C 150 rpm shaking until depletion of the carbon source, the biomass was harvested, lipids were extracted as described in example X and the fatty acid composition was analyzed as described in example X. The fatty acid compositions of the strains are summarized in Table 12.

Table 12. Fatty acid composition (% of total fatty acid) of strains FS01369, FS01446, FS01447 and FS01448.

Fatty acid	**FS01369**	**FS01446**	**FS01447**	**FS01448**
12:0	0,57	0,55	0,70	0,73
16:0	23,04	21,06	21,66	19,41
Δ9-16:1	19,39	20,01	18,37	20,43
18:0	4,06	3,66	3,69	3,71
Δ9-18:1	20,14	25,02	19,75	29,91
Δ9, Δ12-18:2	18,63	14,86	21,76	13,05
Δ6, Δ9, Δ12-18:3	6,90	3,32	6,18	3,41
Δ8, Δ11, Δ14-20:3	2,52	1,37	2,84	1,80
Δ5, Δ8, Δ11, Δ14-20:4	1,10	0,54	1,13	0,52
Δ9, Δ12, Δ15-18:3	-	4,27	-	2,28
Δ6, Δ9, Δ12, Δ15-18:4	-	0,81	-	0,46
Δ8, Δ11, Δ14, Δ17-20:4	-	0,67	-	0,50
Δ5, Δ8, Δ11, Δ14, Δ17-20:5	-	0,33	-	0,19
Others	3,67	3,52	3,91	3,60
Sum	100	100	100	100

The analysis showed that FS01446 and FS01448, both strains expressing *S. kluyveri FAD3*, produced eicosapentaenoic acid (Table 12, Figure 21). However, expression of mouse *Ssc2* in FS01447 and FS01448 did not result in the expected elongation of 20:4 into 22:4 (or 20:5 into 22:5).

Example 59

Codon-optimization and assembly of synthetic delta-4 desaturase from Thraustochytrium

The sequence of *Thraustochytrium* delta-4 desaturase (SEQ ID NO 35) was codon-optimized for expression in *S. cerevisiae* and was assembled from synthetic oligonucleotides. The prodcedure was as follows:

The *Thraustochytrium* nucleotide sequence encoding a delta-4 desaturase was codon-optimized for expression in *S. cerevisiae* using the Backtranslation tool (Entelechon) with the "discard codons below 50% theroretical ratio"-option. The codon-optimized gene (SEQ ID NO 84) was then assembled from chemically synthesized oligo nucleotides. The method used for assembly of the gene is shown in Figure 22. Sense- and antisense oligonucleotides were designed to cover the complete sequence of the gene, and were designed in a way so that a 20 bp overlap was achieved for each complementary sense and antisense oligonucleotide (Figure 22). The sense oligonucleotides were each 40 bp of length and were number D4D-A01 to D4D-A39 consecutively from the 5' end to the 3' end of the sense strand. Similarly, the antisense oligonucleotides were numbered D4D-B01 to D4D-40 consecutively from the 5' end to the 3' end of the antisense strand. First, 200 bp-pieces of double stranded DNA were assembled by mixing five sense oligonucleotides with their 5 complementary antisense oligonucleotides. For example, 100 pmol of each of the oligonucleotides D4D-01, D4D-02, D4D-03, D4D-04 and D4D-05, D4D-B40, D4D-39, D4D-38, D4D-37 and D4D-36 were mixed in a volume of 50 µl. The oligonucleotide mix was then loaded on a 2% agarose gel together with a size marker, and the smear around the size of 200 bp was excised from the gel and was purified. The purified mix was then used as template in a PCR reaction using oligonucleotides D4D-A01 and D4D-B36 as primers, which resulted in amplification of the desired 200 bp fragment, named D4D-1. In analogy, the remaining part of the gene was assembled in 200 bp pieces, named D4D-2 to D4D-8 (Figure 22). Next, the eight 200 bp-fragments were fused by PCR to four fragments of 400 bp, named D4D-12, D4D-34, D4D-56 and D4D-78. These fragments were then fused by PCR to form two fragments of 800 bp, and finally the two 800 bp-fragments were fused to form the full gene. The final fusion PCR reaction was carried out using the primers 5' ATCCCGGGACCATGACAGTTGGTTACGATGAGG 3' and 5' ATCCGCGGTTATGCTGCTCTTTGCCAACTTTCG 3', which introduced XmaI and SacII restriction sites at the 5' end and 3' end of the gene, respectively.

Example 60

Cloning of synthetic delta-4 desaturase into a yeast expression vector

The synthetically assembled gene encoding delta-4 desaturase (Example 59) was digested with XmaI and SacII and was introduced into XmaI/SacII digested pESC-LEU-Ssc2, resulting in the plasmid pESC-LEU-Ssc2-delta-4d (Figure 23).

Example 61

Integration of S. kluyveri FAD3 into the genome of S. cerevisiae

The *S. kluyveri FAD3* gene, encoding an omega-3 desaturase was integrated into the genome of *S. cerevisiae* and was placed under the control of the yeast *GAL1* promoter. The *GAL1* promoter and the *S. kluyveri FAD3* gene were integrated at the locus of *GPP1*, resulting in knockout of this gene (see also Example 66). The integration was carried out through homologous recombination using a bipartite gene targeting substrate (Figure 24). One part of the bipartite substrate consisted of two thirds (towards the 3´end) of *K. lactis URA3*, fused to the *GAL1* promoter sequence, the *S.kluyveri FAD3* gene and a target sequence downstream of *GPP1*. The second part of the bipartite substrate consisted of a target sequence upstream of *GPP1*, fused to the *GAL1* promoter sequence and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants were obtained in which *GPP1* had been knocked out and replaced with two copies of the *GAL1* promoter sequence as a direct repeat on either side of the *K. lactis* URA3 marker gene and the *S.kluyveri FAD3* gene immediately downstream of the second *GAL1* promoter repeat. A second recombination event, resulting in looping out of the selection marker, was selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the URA3 gene. This resulted in a strain, in which the *GPP1* gene had been replaced with the *S.kluyveri FAD3* under the control of the *GAL1* promoter.

The procedure was as follows:

For construction of the first part of the bipartite gene targeting substrate, a fragment containing the *GAL1* promoter immediately upstream of *S. kluyveri FAD3* was amplified from plasmid pESC-TRP-SK33 (see example X for construction of pESC-TRP-SK33), using the primers 5' ACTACATCATCGAATTCCAGAACGAATCAAATTAACAACCATAG 3' and 5' TCATTGACTGGAACCATCTT 3'. A target sequence downstream of *S. cerevisiae GPP1* was amplified by PCR using *S. cerevisiae* genomic DNA as template and the primers 5´ AAGATGGTTCCAGTCAATGATAAGGATGACTTGTTGAAATGGTAA 3´ and 5´CCACAAGACTGTTTCCAGAGC 3´. A third DNA fragment, consisting of two-thirds of *K. lactis URA3* towards the 3' end, was generated by PCR using as template a plasmid containing the *K. lactis URA3* and the primers 5´ CTTGACGTTCGTTCGACTGATGAGC 3´ and 5´CTGGAATTCGATGATGTAGTTTCTGG 3´. These PCR fragments were then fused during two rounds of PCR. First, the fragment containing the *GAL1* promoter and the *S. kluyveri FAD3* was fused to the downstream target sequence using the primers 5´

ACTACATCATCGAATTCCAGAACGAATCAAATTAACAACCATAG 3´ and 5´ CCACAAGACTGTTTCCAGAGC 3´. Second, the product of the first fusion reaction was fused to the 3' 2/3 *K. lactis URA3* fragment using the primers 5´ CTTGACGTTCGTTCGACTGATGAGC 3´ and 5´ CCACAAGACTGTTTCCAGAGC 3´. This resulted in the fusion product 2/3*URA3*-p*GAL1-S.kluyveri FAD3*-DOWN, which constituted the first part of the bipartite gene targeting substrate.

For construction of the second part of the bipartite substrate, a target sequence upstream of *GPP1* was amplified by PCR using *S. cerevisiae* genomic DNA as template and the primers 5´ ATGGCATGGCCCCGAAGG 3´ and 5´ CTGGAATTCGATGATGTAGTTTGAACGAAAATGAACAAGACG 3´. The *GAL1* promoter was amplified by PCR using pESC-TRP-SK33 as template and the primers 5' ACTACATCATCGAATTCCAGAACGAATCAAATTAACAACCATAG 3' and 5' CCACCATCCAATGCAGACCGCGGGGTTTTTTCTCCTTGAC 3'. A third fragment, consisting of two thirds of *K. lactis URA3* towards the 5' end, was generated by PCR using the primers 5´ CGGTCTGCATTGGATGGTGGTAAC 3' and 5' GAGCAATGAACCCAATAACGAAATC 3', and a plasmid containing the *K. lactis URA3* as template. These PCR fragments were then fused during two rounds of PCR. First, the *GAL1* promoter was fused to the upstream targeting sequence using the primers 5' ATGGCATGGCCCCGAAGG 3' and 5' CCACCATCCAATGCAGACCGCGGGGTTTTTTCTCCTTGAC 3'. Second, the product of the first fusion reaction was fused to the 5' 2/3 *K. lactis URA3* fragment using the primers 5´ ATGGCATGGCCCCGAAGG 3' and 5' GAGCAATGAACCCAATAACGAAATC 3'. This resulted in the fusion product UP- p*GAL1*-2/3*URA3*, which constituted the second part of the bipartite gene targeting substrate.

The yeast strain FS01444 (MATalpha ura3 trp1 leu2 pox1::pTDH3-mole1, Example 57) is transformed with the linear substrates 2/3*URA3*-p*GAL1-S.kluyveri FAD3*-DOWN and UP-p*GAL1*-2/3*URA3* and plated out on medium lacking uracil. Transformants are streak purified on the same medium and then transferred onto medium containing 5-FOA. Pop-out recombinants are streak purified on 5-FOA-containing medium and are verified by colony PCR. The resulting strain has the genotype MATalpha ura3 trp1 leu2 pox1::pTDH3-mole1 gpp1::pGAL1-*S.kluyveri FAD3* and is named FS01460.

Example 62

Expression of the pathway to docosahexaenoic acid

To express the full pathway to docosahexaenoic acid, FS01460 (MATalpha ura3 trp1 leu2 pox1::pTDH3-mole1 gpp1::pGAL1-*S.kluyveri FAD3*, Example 61) is co-transformed with

the plasmids pESC-TRP-delta-12 delta-6, pESC-URA-elo-delta-5 and pESC-LEU-Ssc2-delta-4d. Transformants are selected and streak-purified on medium lacking uracil, tryptophane and leucine. The transformed strain is then cultivated under suitable conditions for induction of the GAL promoters (e.g. Example 9, Example 49), and the fatty acid composition is analyzed as described in Example 45.

Example 63

Overexpression of LRO1

The acyltransferase encoded by *LRO1* in *S. cerevisiae* catalyses the transfer of an acyl chain from position 2 in phosphatidylcholine to diacylglycerol, resulting in the formation of triacylglycerol. Since polyunsaturated fatty acid desaturation takes place mainly on position 2 of phosphatidylcholine (Domergue, F. et al. (2003) J Biol Chem 278: 35115-35126), overexpression of *LRO1* is likely to result in increased incorporation of polyunsaturated fatty acid into triacylglycerol and overall increased polyunsaturated fatty acid content. *LRO1* is overexpressed with a strong yeast promoter, for example the *TDH3*, *ADH1*, *TPI1* or *HXT7* promoter using a using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15)), as described in e.g. Example 26 and Example 30.

Example 64

Overexpression of ARE1 and ARE2

The acyltransferases encoded by *ARE1* and *ARE2* in *S. cerevisiae* catalyze the addition of an acyl chain to diacylglycerol to form triacylglycerol. Overexpression of *ARE1* and *ARE2* may result in increased lipid yield and overall increased polyunsaturated fatty acid content. *ARE1* and *ARE2* are overexpressed with strong yeast promoters, for example the *TDH3*, *ADH1*, *TPI1* or *HXT7* promoters using a using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15) , as described in e.g. Example 26 and Example 30.

Example 65

Overexpression of ELO1, ELO2 and ELO3

The yeast genes *ELO1, ELO2* and *ELO3* encode fatty acid elongases. The elongase encoded by *ELO1* is responsible for elongation of C14 fatty acids to C16 species (Toke et al. (1996) J Biol Chem 271: 18413-18422), ELO2 encodes an elongase involved in synthesis of saturated and monounsaturated fatty acid of up to 24 carbon atoms in length, and ELO3

encodes an elongase with a broad substrate range (Oh, C.-S. et al. (1997) J Biol Chem 272: 17376-17384). Overexpression of these elongases may contribute to increased contents of C18 fatty acids, the substrates for the polyunsaturated fatty acid pathway, and thereby result in increased production of polyunsaturated fatty acids. *ELO1, ELO2* and *ELO3* are overexpressed with strong yeast promoters, for example the *TDH3*, *ADH1*, *TPI1* or *HXT7* promoters using a using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15), as described in e.g. Example 26 and Example 30.

Example 66

Overexpression of GPD1 and deletion of GPP1 and GPP2

The universal precursor for lipid synthesis is glycerol-3-phosphate, which is formed from dihydroxyacetonephosphate by the action of NAD-dependent glycerol-3-phosphate dehydrogenase, in yeast encoded by *GPD1*. Glycerol-3-phosphate either enter the lipid synthesis pathway or it can be dephosphorylated to form glycerol by the action of glycerol-3-phosphatases encoded by *GPP1* and *GPP2*. By overexpressing GPD1 and deleting GPP1 and GPP2, the availability of glycerol-3-phosphate can be increased (Nguyen, H.T.T. et al. (2004) Metab Eng. *6*,155-163). By combining these modifications with overexpression of the FAS complex and acyltransferases in the lipid synthesis pathway, it is likely that the lipid yield and polyunsaturated fatty acid yield can be improved.

GPD1 is overexpressed with a strong yeast promoter, for example the *TDH3*, *ADH1*, *TPI1* or *HXT7* promoter, using a using a promoter-replacement method based on a bipartite gene-targeting substrate (Figure 15) , as described in e.g. Example 26 and Example 30.

GPP1 and *GPP2* are deleted using a bipartite gene-targeting substrate with *K.lactis URA3* as a recyclable marker. One part of the bipartite substrate consists of two thirds (towards the 3´end) of *K. lactis URA3*, followed by a short sequence R, and a target sequence downstream of *GPP1* or *GPP2*, respectively. The second part of the bipartite substrate consists of a target sequence upstream of *GPP1* or *GPP2*, respectively, fused to the short sequence R and two thirds (towards the 5´ end) of *K. lactis URA3*. Following transformation with the bipartite substrate and selection on medium lacking uracil, transformants are obtained in which *GPP1* or *GPP2*, respectively, have been knocked out and replaced with two copies of the short sequence R as a direct repeat on either side of the *K. lactis* URA3 marker gene. A second recombination event, resulting in looping out of the selection marker, is selected for by replating transformants on medium containing 5´-fluoroorotic acid (5-FOA), which is toxic to cells expressing the URA3 gene. In the resulting

strains, *GPP1* and *GPP2* have been deleted and replaced with the short sequence R. Alternatively, *GPP1* and *GPP2* are used as the loci for integration of heterologous genes, resulting in deletion of these genes, e.g. as described in Example 66.

The *GPD1* overexpression and the *GPP1* and *GPP2* deletions are performed in a *pADH1-FAS1 pADH1-FAS2 pTPI1-ACC1 pox1::pTDH3-M.alpina ole1* strain background. Following construction of the *GPD1* overexpression, *GPP1* and *GPP2* deletion strain, these modifications are combined with overexpression of acyltransferases in the lipid biosynthetic pathway (e.g. overexpression of *GAT1*, *SLC1* and *DGA1* or *LRO1*) through crossing of strains.

Example 67

Expression of a heterologous NADP$^+$ dependent glyceraldehyde 3-phosphate dehydrogenase

Expression of the *Streptococcus mutans GapN* gene (SEQ ID NO: 91), encoding an NADP$^+$ dependent glyceraldehyde 3-phosphate dehydrogenase, increases the availability of cytosolic NADPH for fatty acid synthesis. Integration of the *S. mutans GapN* into the genome of *S. cerevisiae* is combined with overexpression of *FAS1*, *FAS2* and *ACC1*.

The *S. mutans GapN* gene is integrated into the genome of *S. cerevisiae* and is placed under the control of the yeast *ADH1* promoter. The *ADH1* promoter and the *S. mutans GapN* gene are integrated at the locus of *GPP2*, resulting in knockout of this gene (see also Example 66). The integration is carried out through homologous recombination using a bipartite gene targeting substrate, using the same principle methods as described for the integration of *M. alpina ole1* (Example 28)..

Example 68

Combining genetic modifications in a single strain

Genetic modifications that lead to improved lipid yields and/or polyunsaturated fatty acid yields are combined by crossing of strains or by repeating the promoter replacement procedure in different strain backgrounds. For example, overexpression of *FAS1*, *FAS2*, and *ACC1* is combined with overexpression of *GAT1*, *SLC1*, and *DGA1* and/or *LRO1*.

Example 69

Codon-optimization and assembly of synthetic delta-9 elongase from Isochrysis galbana and synthetic delta-8 desaturase from Euglena gracilis

The sequences encoding *Isochrysis galbana* delta-9 elongase (SEQ ID NO 37) and *Euglena gracilis* delta-8 desaturase (SEQ ID NO 38) are codon-optimized for expression in *S. cerevisiae* using the Backtranslation tool (Entelechon) with the "discard codons below 50% theroretical ratio"-option. The codon-optimized genes (SEQ ID NO 85 and SEQ ID NO 86, respectively) are then assembled from chemically synthesized oligo nucleotides, in principle as described for the assembly of a synthetic gene encoding delta-4 desaturase (Example 59).

Example 70

Cloning of synthetic delta-9 elongase and delta-8 desaturase into yeast expression vectors

The codon-optimized gene encoding a delta-9 elongase (SEQ ID NO 85, Example 70) is cloned into the yeast expression vector pESC-TRP-delta-12 (Example 2), resulting in the vector pESC-TRP-delta-12 delta-9e. Furthermore, the vectors pESC-URA-elo-delta-5 is digested with suitable restriction enzymes resulting in the release of the *M.alpina* delta-6 elongase encoding gene from this plasmid. The linearized plasmid is purified and the codon-optimized delta-8 desaturase (SEQ ID NO 86, Example 71) is introduced in the place of the *M.alpina* delta-6 elongase. The resulting plasmid is named pESC-URA-delta-8 delta-5.

Example 71

Expression of the pathway to arachidonic acid via codon-optimized delta-9 elongase and delta-8 desaturase

A suitable yeast strain (e.g. FS01267, FS01368, FS01408 or FS01423) is co-transformed with plasmids pESC-TRP-delta-12 delta-9e and pESC-URA-delta-8 delta-5. The resulting strain is cultivated under suitable conditions for induction of the GAL promoters (e.g. Example 9, Example 49), and the fatty acid composition is analyzed as described in Example 45.

Example 72

Expression of the pathway to eicosapentaenoic acid via codon-optimized delta-9 elongase and delta-8 desaturase

A suitable yeast strain (e.g. FS01277 or FS01444) is co-transformed with plasmids pESC-TRP-delta-12 delta-9e (Example 70), pESC-URA-delta-8 delta-5 (Example 70) and pESC-LEU-SK33 (Example 54). The resulting strain is cultivated under suitable conditions for induction of the GAL promoters (e.g. Example 9, Example 49), and the fatty acid composition is analyzed as described in Example 45.

Example 73

Expression of the pathway to docosatetraenoic acid via codon-optimized delta-9 elongase and delta-8 desaturase

A suitable yeast strain (e.g. FS01277 or FS01444) is co-transformed with plasmids pESC-TRP-delta-12 delta-9e (Example 70), pESC-URA-delta-8 delta-5 (Example 70) and pESC-LEU-Ssc2 (Example 55). The resulting strain is cultivated under suitable conditions for induction of the GAL promoters (e.g. Example 9, Example 49), and the fatty acid composition is analyzed as described in Example 45.

Example 74

Expression of the pathway to docosapentaenoic acid via codon-optimized delta-9 elongase and delta-8 desaturase

A suitable yeast strain (e.g. FS01277 or FS01444) is co-transformed with plasmids pESC-TRP-delta-12 delta-9e (Example 70), pESC-URA-delta-8 delta-5 (Example 70) and pESC-LEU-Ssc2-SK33 (Example 56). The resulting strain is cultivated under suitable conditions for induction of the GAL promoters (e.g. Example 9, Example 49), and the fatty acid composition is analyzed as described in Example 45.

Example 75

Expression of the pathway to docosahexaenoic acid via codon-optimized delta-9 elongase and delta-8 desaturase

The yeast strain FS01460 (Example 61) is co-transformed with plasmids pESC-TRP-delta-12 delta-9e (Example 70), pESC-URA-delta-8 delta-5 (Example 70) and pESC- pESC-LEU-

Ssc2-delta-4d (Example 60). The resulting strain is cultivated under suitable conditions for induction of the GAL promoters (e.g. Example 9, Example 49), and the fatty acid composition is analyzed as described in Example 45.

Example 76

Codon-optimization of genes encoding delta-9 desaturase, delta-12 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, delta-5 elongase and omega-3 desaturase

The sequences of *M. alpina* delta-9 desaturase (SEQ ID NO 1), delta-12 desaturase (SEQ ID NO 5), delta-6 desaturase (SEQ ID NO 11), delta-6 elongase (SEQ ID NO 16) and delta-5 desaturase (SEQ ID NO 22), mouse delta-5 elongase (SEQ ID NO 28) and *S. kluyveri* omega-3 desaturase (SEQ ID NO 87) are codon-optimized for expression in *S. cerevisiae* and are assembled from synthetic oligonucleotides using the same principle as described for assembly of a synthetic gene encoding delta-4 desaturase (Example 59).

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope thereof.

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Claims

1. A method for producing polyunsaturated fatty acids with four or more double bonds comprising heterologous expression of an oxygen requiring pathway in a *Saccharomyces cerevisiae* grown on a non-fatty acid substrate.

2. A method according to claim 1, wherein said non-fatty acid substrate is the exclusive carbon source.

3. A method according to any of claims 1-2, wherein the heterologous expression comprises combining heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase.

4. A method according to any of claims 1-2, wherein the heterologous expression comprises combining heterologous expression of nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase.

5. A method according to any of the preceding claims, wherein the combined heterologous expression further comprises heterologous expression of a nucleotide sequence encoding delta-5 elongase, omega-3 desaturase, and/or delta-4 desaturase.

6. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises heterologous expression of a nucleotide sequence encoding a delta-9 desaturase.

7. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises an over-expression of at least one of the genes selected from the group consisting of ACC1, YBR159W, ELO1, ELO2, ELO3, FAS1, FAS2, DGA1, LRO1, ARE1, ARE2, and GPD1.

8. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises a deletion of at least one of the genes selected from the group consisting of GPP1, GPP2 and POX1.

9. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises a heterologous expression of the nucleotide sequences encoding ATP:citrate lyase and/or an isocitrate dehydrogenase which is stimulated by AMP.

10. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises a heterologous expression of a nucleotide sequence encoding a non-phosphorylating NADP-dependent D-glyceraldehyde-3-phosphate dehydrogenase.

11. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises a deletion of the gene GDH1 and optionally an over-expression of at least one of the genes selected from the group consisting of GDH2, GLN1 and GLT1.

12. A method according to any of the preceding claims, wherein said combined heterologous expression further comprises an over-expression of at least one of the genes selected from the group consisting of TSC13, GAT1, SLC1 and YDR531W.

13. A method according to any of the preceding claims, wherein said heterologous nucleotide sequences are codon optimized for expression in *Saccharomyces cerevisiae*.

14. A method according to any of the preceding claims, wherein said *Saccharomyces cerevisiae* is cultivated in a myo-inositol deficient medium.

15. A method according to any of the preceding claims, wherein the polyunsaturated fatty acid is selected from the group consisting of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.

16. A method according to any of the preceding claims, wherein said heterologous expression increases the content of arachidonic acid, eicosapentaenoic acid and/or docosahexaenoic acid to more than 2 % of the total fatty acid content in said *Saccharomyces cerevisiae*

17. A method according to any of claims 3-16, wherein the nucleotide sequence encoding delta-12 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NOs 5-10, 93, 95, and 113; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NOs 5-10, 93, 95, and 113.

18. A method according to any of claims 4-17, wherein the nucleotide sequence encoding delta-9 elongase is a nucleotide sequence comprising or having at least 75% identity to the nucleotide sequence of SEQ ID NO: 37.

19. A method according to any of claims 6-18, wherein the nucleotide sequence encoding delta-9 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 1-4; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 1-4.

20. A method according to any of claims 4-19, wherein the nucleotide sequence encoding delta-8 desaturase is a nucleotide sequence comprising or having at least 75% identity to the nucleotide sequence of SEQ ID NO: 38.

21. A method according to any of claims 3-17 or 19, wherein the nucleotide sequence encoding delta-6 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, and 99; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 11-15, 97, and 99.

22. A method according to any of claims 3-17, 19 or 21, wherein the nucleotide sequence encoding delta-6 elongase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 16-21, 101 and 103; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 16-21, 101 and 103.

23. A method according to any of claims 3-22, wherein the nucleotide sequence encoding delta-5 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105 and 107; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 22-27, 99, 105 and 107.

24. A method according to any of claims 3-23 wherein the nucleotide sequence encoding delta-5 elongase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 19, 28, 29 and 101;

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 19, 28, 29 and 101; and

c) nucleotide sequences encoding amino acid sequences that have at least 75% identity to SEQ ID NO 68.

25. A method according to any of claims 3-24, wherein the nucleotide sequence encoding delta-4 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 35-36 and 109;

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 35-36 and 109; and

c) nucleotide sequences encoding amino acid sequences that have at least 75% identity to SEQ ID NOs 76-77.

26. A method according to any of claims 2-25, wherein the nucleotide sequence encoding omega-3 desaturase is selected from the group consisting of

a) the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89 and 111; and

b) nucleotide sequences having at least 75% identity to the nucleotide sequences set forth in SEQ ID NO: 30-34, 87, 89 and 111.

27. A method for producing a polyunsaturated fatty acid comprising the steps of

(a) isolating at least 4 nucleotide sequences encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase, and delta-5 desaturase

(b) constructing one or more vectors comprising said isolated nucleotide sequences of step (a) and/or integrating said isolated nucleotide sequences into the genome of *Saccharomyces cerevisiae*;

(c) optionally, transforming said vector(s) of step (b) into a *Saccharomyces cerevisiae* for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a);

(d) growing said *Saccharomyces cerevisiae* on a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product

and obtaining said polyunsaturated fatty acid.

28. A method for producing a polyunsaturated fatty acid comprising the steps of

(a) isolating at least 4 nucleotide sequences encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase, and delta-5 desaturase

(b) constructing one or more vectors comprising said isolated nucleotide sequences of step (a) and/or integrating said isolated nucleotide sequences into the genome of *Saccharomyces cerevisiae*;

(c) optionally, transforming said vector(s) of step (b) into a *Saccharomyces cerevisiae* for a time and under conditions sufficient for expression of proteins encoded by said isolated nucleotide sequences of step (a);

(d) growing said *Saccharomyces cerevisiae* on a non-fatty acid substrate, whereby said non-fatty acid substrate is converted by said host into a desired polyunsaturated fatty acid product

and obtaining said polyunsaturated fatty acid.

29. A method according to any of claims 27-28, wherein the heterologous expression further comprises heterologous expression of a nucleotide sequence encoding delta-9 desaturase, delta-5 elongase, omega-3 desaturase, and/or delta-4 desaturase.

30. A genetically modified *Saccharomyces cerevisiae* capable of producing polyunsaturated fatty acids with four or more double bonds when grown on a non-fatty acid substrate.

31. A genetically modified *Saccharomyces cerevisiae* according to claim 30, wherein said Saccharomyces cerevisiae is capable of producing polyunsaturated fatty acids with four or more double bonds rown on a non-fatty acid substrate as the exclusive carbon source.

32. A composition comprising a polyunsaturated fatty acid produced by a genetically modified *Saccharomyces cerevisiae* according to any of claims 30-31.

33. A composition comprising at least 25% polyunsaturated fatty acid in total fatty acid composition produced by a genetically modified cell according to any of claims 30-31.

34. A composition according to any of claims 32-33, wherein said composition is an oil.

35. A composition according to any of claims 32-33, wherein said polyunsaturated fatty acid is incorporated in triacylglycerides.

36. A composition according to any of claims 32-33, wherein said polyunsaturated fatty acids is incorporated in phospholipids.

37. A composition according to any of claims 32-33, wherein said polyunsaturated fatty acids is in a form of free fatty acids.

38. Use of a composition according to any of claims 32-37 as an ingredient in a food product.

39. Use of a composition according to any of claims 32-37 as an ingredient in a cosmetic product.

40. Use of a composition according to any of claims 32-37 as an ingredient in feed.

41. Use of a genetically modified *Saccharomyces cerevisiae* according to claim 29 as an ingredient in feed.

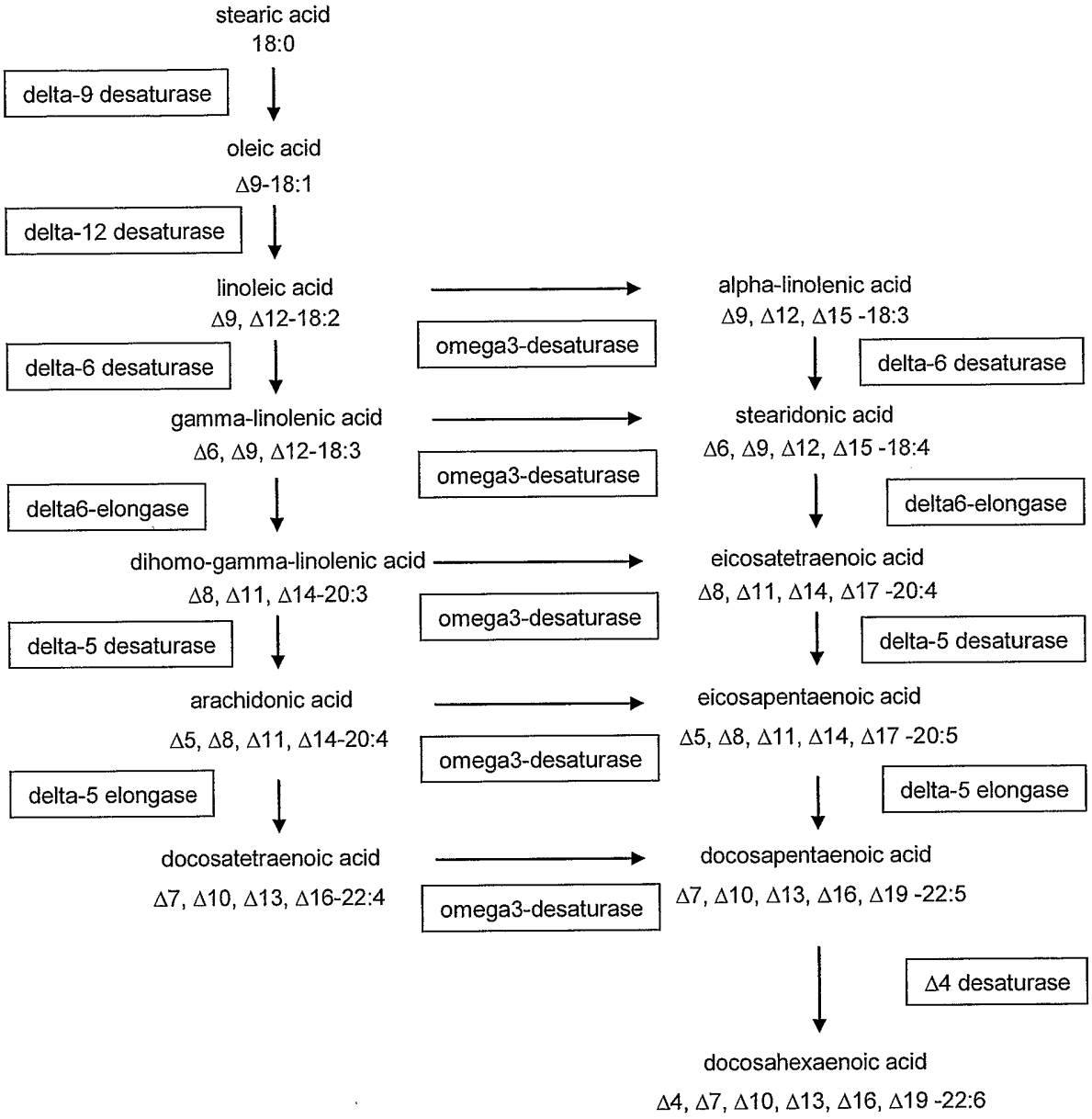

stearic acid
18:0
delta-9 desaturase
oleic acid
Δ9-18:1
delta-12 desaturase
linoleic acid
Δ9, Δ12-18:2
omega3-desaturase
alpha-linolenic acid
Δ9, Δ12, Δ15 -18:3
delta-6 desaturase
delta-6 desaturase
gamma-linolenic acid
Δ6, Δ9, Δ12-18:3
omega3-desaturase
stearidonic acid
Δ6, Δ9, Δ12, Δ15 -18:4
delta6-elongase
delta6-elongase
dihomo-gamma-linolenic acid
Δ8, Δ11, Δ14-20:3
omega3-desaturase
eicosatetraenoic acid
Δ8, Δ11, Δ14, Δ17 -20:4
delta-5 desaturase
delta-5 desaturase
arachidonic acid
Δ5, Δ8, Δ11, Δ14-20:4
omega3-desaturase
eicosapentaenoic acid
Δ5, Δ8, Δ11, Δ14, Δ17 -20:5
delta-5 elongase
delta-5 elongase
docosatetraenoic acid
Δ7, Δ10, Δ13, Δ16-22:4
omega3-desaturase
docosapentaenoic acid
Δ7, Δ10, Δ13, Δ16, Δ19 -22:5
Δ4 desaturase
docosahexaenoic acid
Δ4, Δ7, Δ10, Δ13, Δ16, Δ19 -22:6

Fig. 1

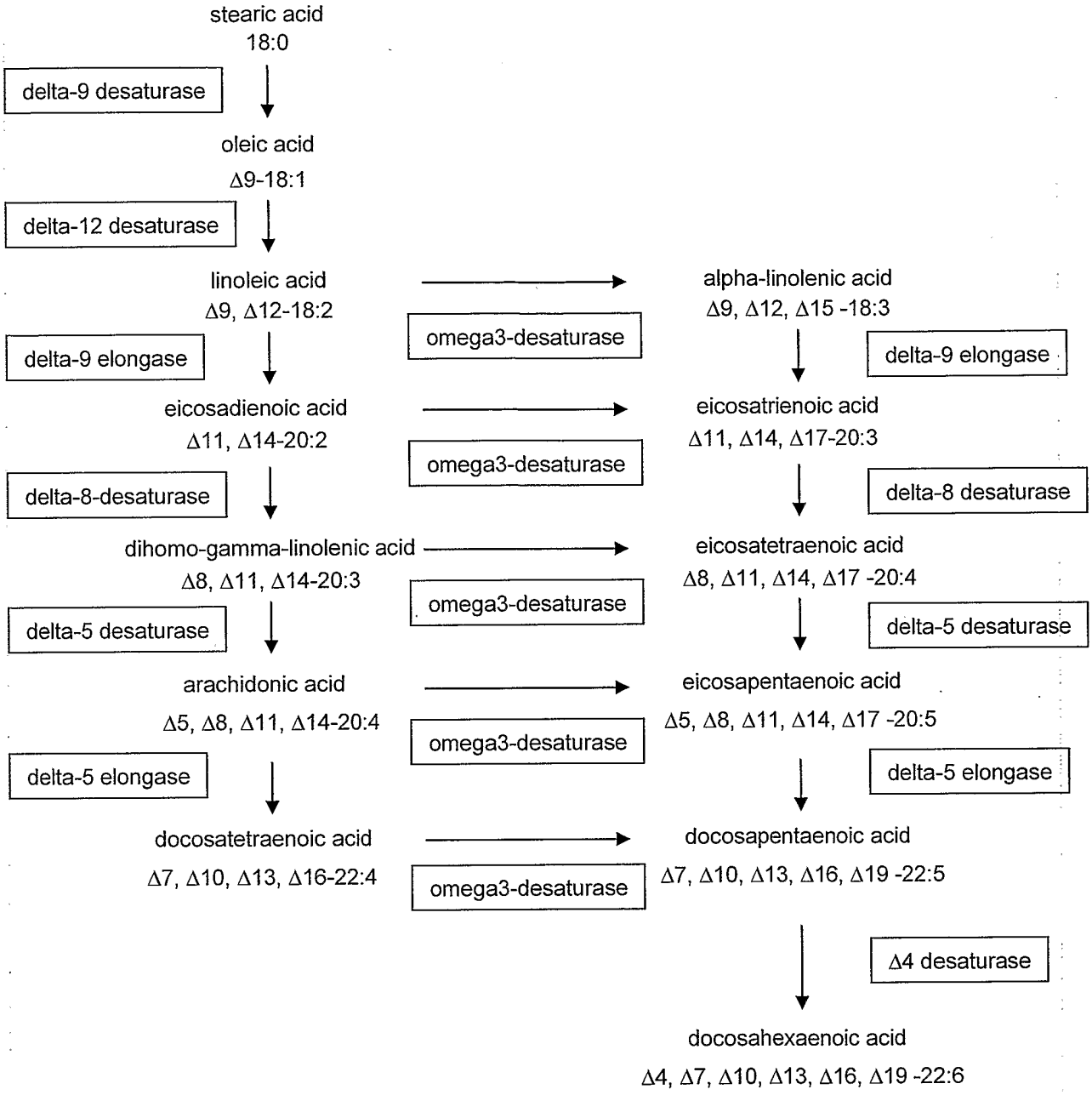

stearic acid
18:0
delta-9 desaturase
oleic acid
Δ9-18:1
delta-12 desaturase
linoleic acid
Δ9, Δ12-18:2
omega3-desaturase
alpha-linolenic acid
Δ9, Δ12, Δ15 -18:3
delta-9 elongase
delta-9 elongase
eicosadienoic acid
Δ11, Δ14-20:2
omega3-desaturase
eicosatrienoic acid
Δ11, Δ14, Δ17-20:3
delta-8-desaturase
delta-8 desaturase
dihomo-gamma-linolenic acid
Δ8, Δ11, Δ14-20:3
omega3-desaturase
eicosatetraenoic acid
Δ8, Δ11, Δ14, Δ17 -20:4
delta-5 desaturase
delta-5 desaturase
arachidonic acid
Δ5, Δ8, Δ11, Δ14-20:4
omega3-desaturase
eicosapentaenoic acid
Δ5, Δ8, Δ11, Δ14, Δ17 -20:5
delta-5 elongase
delta-5 elongase
docosatetraenoic acid
Δ7, Δ10, Δ13, Δ16-22:4
omega3-desaturase
docosapentaenoic acid
Δ7, Δ10, Δ13, Δ16, Δ19 -22:5
Δ4 desaturase
docosahexaenoic acid
Δ4, Δ7, Δ10, Δ13, Δ16, Δ19 -22:6

Fig. 2

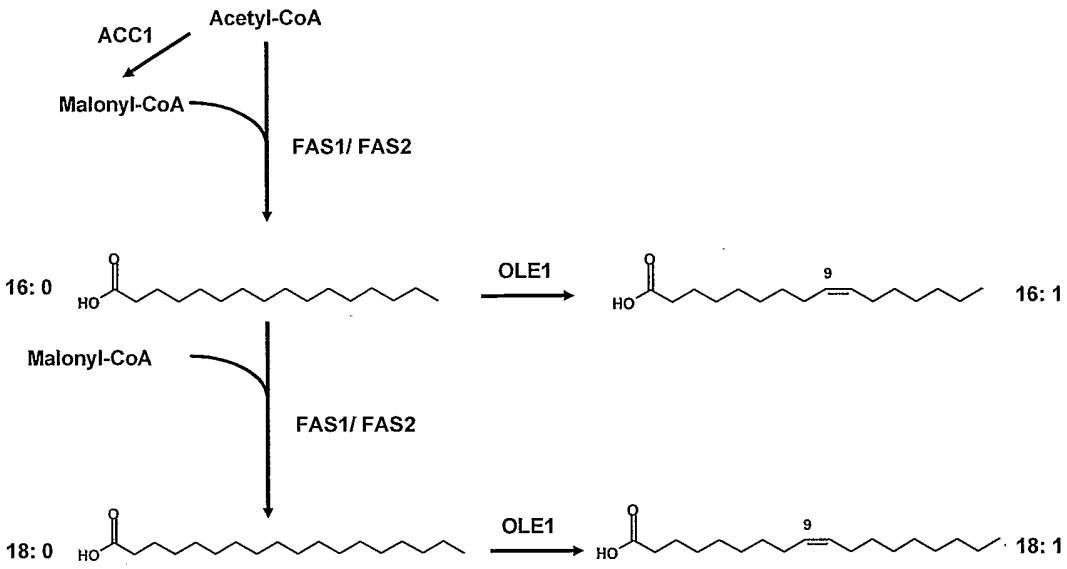

ACC1
Acetyl-CoA
Malonyl-CoA
FAS1/ FAS2
16: 0
HO
OLE1
9
HO
16: 1
Malonyl-CoA
FAS1/ FAS2
18: 0
HO
OLE1
9
HO
18: 1

Fig. 3

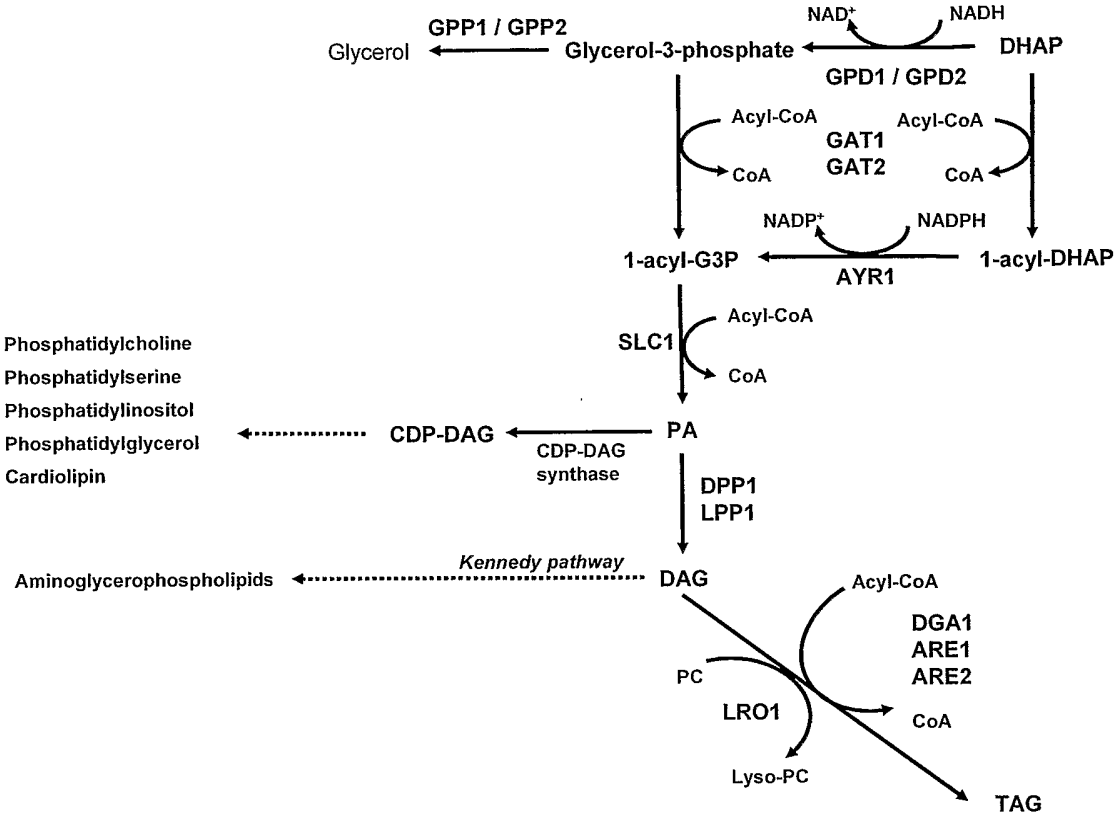

GPP1 / GPP2
Glycerol
Glycerol-3-phosphate
NAD+
NADH
DHAP
GPD1 / GPD2
Acyl-CoA
GAT1
GAT2
Acyl-CoA
CoA
CoA
NADP+
NADPH
1-acyl-G3P
AYR1
1-acyl-DHAP
Acyl-CoA
SLC1
CoA
Phosphatidylcholine
Phosphatidylserine
Phosphatidylinositol
Phosphatidylglycerol
Cardiolipin
CDP-DAG
CDP-DAG synthase
PA
DPP1
LPP1
Kennedy pathway
Aminoglycerophospholipids
DAG
Acyl-CoA
DGA1
ARE1
ARE2
PC
LRO1
CoA
Lyso-PC
TAG

Fig. 4

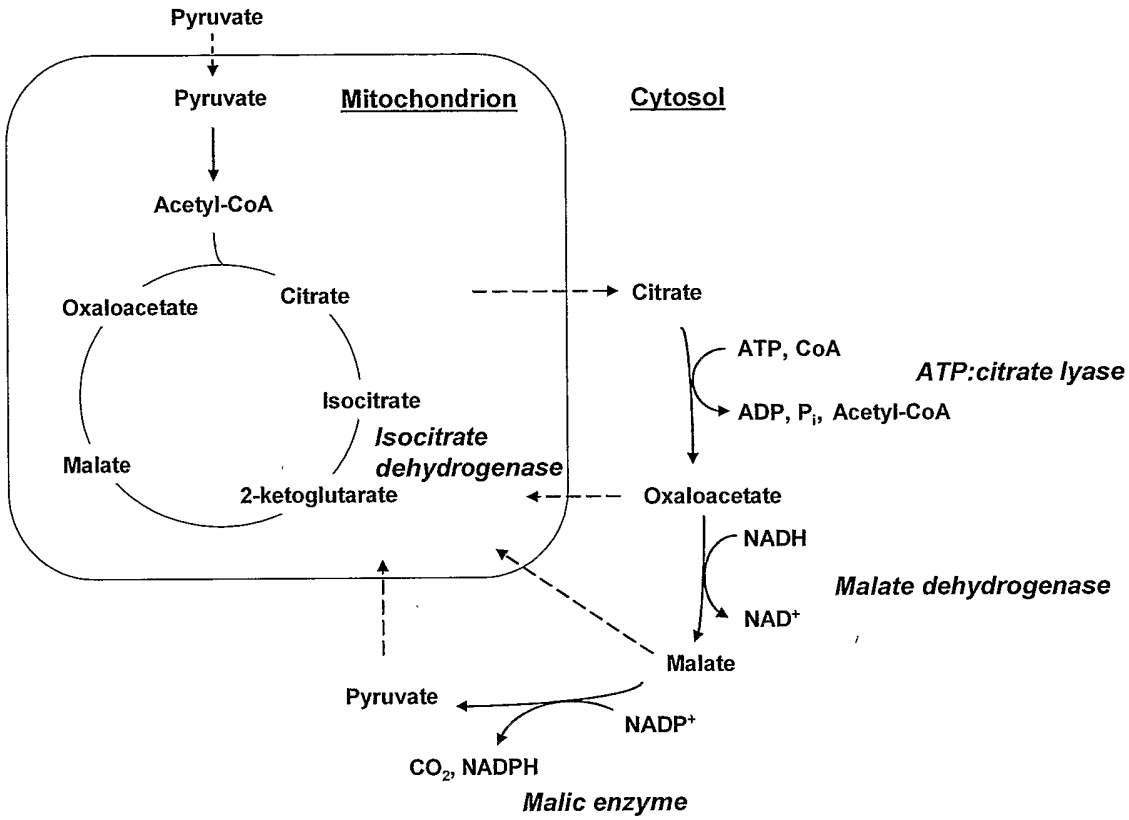

Pyruvate
Pyruvate
Mitochondrion
Cytosol
Acetyl-CoA
Oxaloacetate
Citrate
Citrate
ATP, CoA
ATP:citrate lyase
ADP, Pi, Acetyl-CoA
Isocitrate
Isocitrate dehydrogenase
Malate
2-ketoglutarate
Oxaloacetate
NADH
Malate dehydrogenase
NAD+
Malate
Pyruvate
NADP+
CO2, NADPH
Malic enzyme

Fig. 5

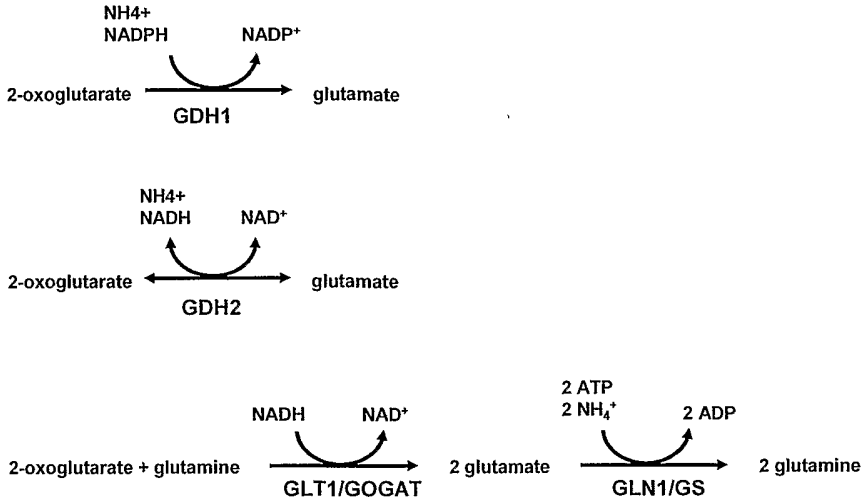

NH4+
NADPH
NADP+
2-oxoglutarate
glutamate
GDH1
NH4+
NADH
NAD+
2-oxoglutarate
glutamate
GDH2
NADH
NAD+
2-oxoglutarate + glutamine
GLT1/GOGAT
2 glutamate
2 ATP
2 NH4+
2 ADP
GLN1/GS
2 glutamine

Fig. 6

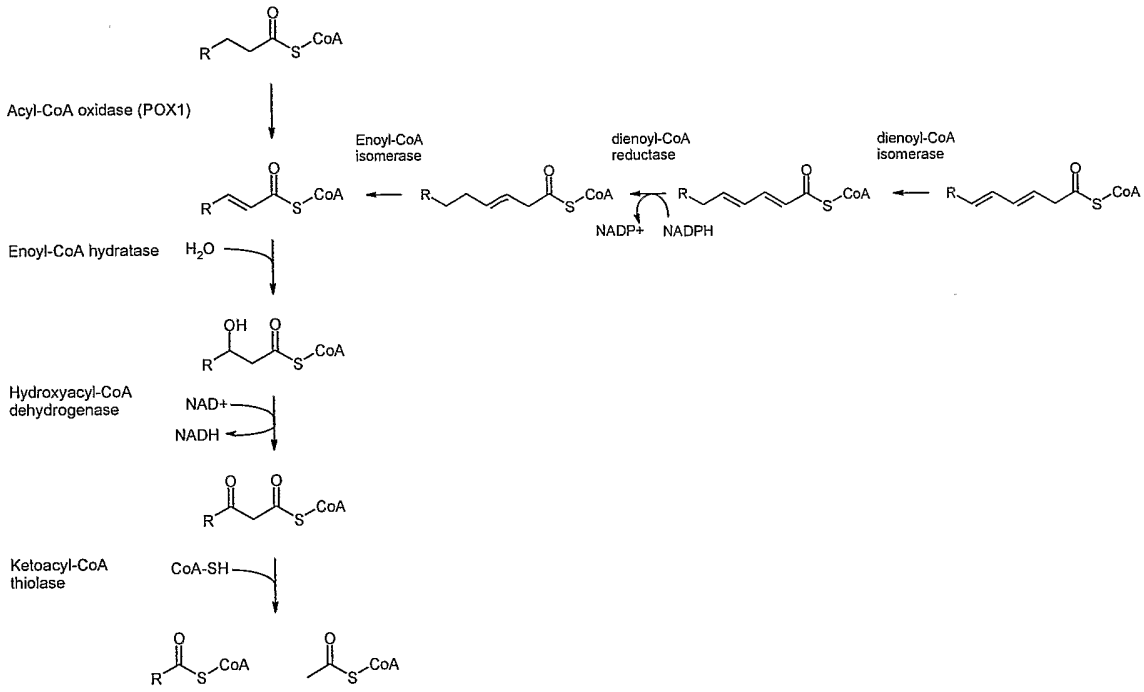

Acyl-CoA oxidase (POX1)
Enoyl-CoA isomerase
dienoyl-CoA reductase
dienoyl-CoA isomerase
NADP+
NADPH
Enoyl-CoA hydratase
H2O
Hydroxyacyl-CoA dehydrogenase
NAD+
NADH
Ketoacyl-CoA thiolase
CoA-SH
CoA
R
OH

Fig. 7

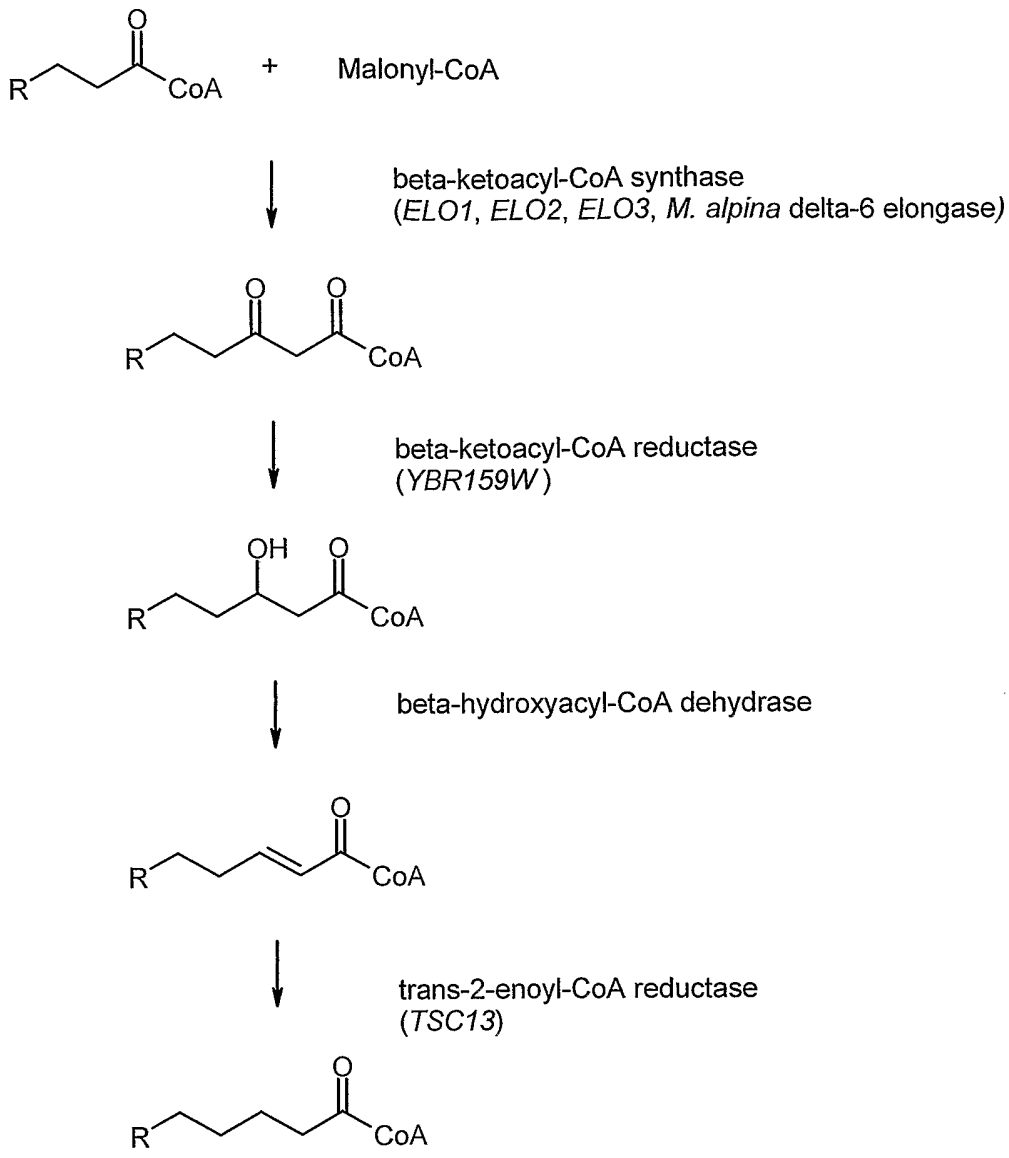

R
O
CoA
+
Malonyl-CoA
beta-ketoacyl-CoA synthase
(ELO1, ELO2, ELO3, M. alpina delta-6 elongase)
R
O
O
CoA
beta-ketoacyl-CoA reductase
(YBR159W)
R
OH
O
CoA
beta-hydroxyacyl-CoA dehydrase
R
O
CoA
trans-2-enoyl-CoA reductase
(TSC13)
R
O
CoA

Fig. 8

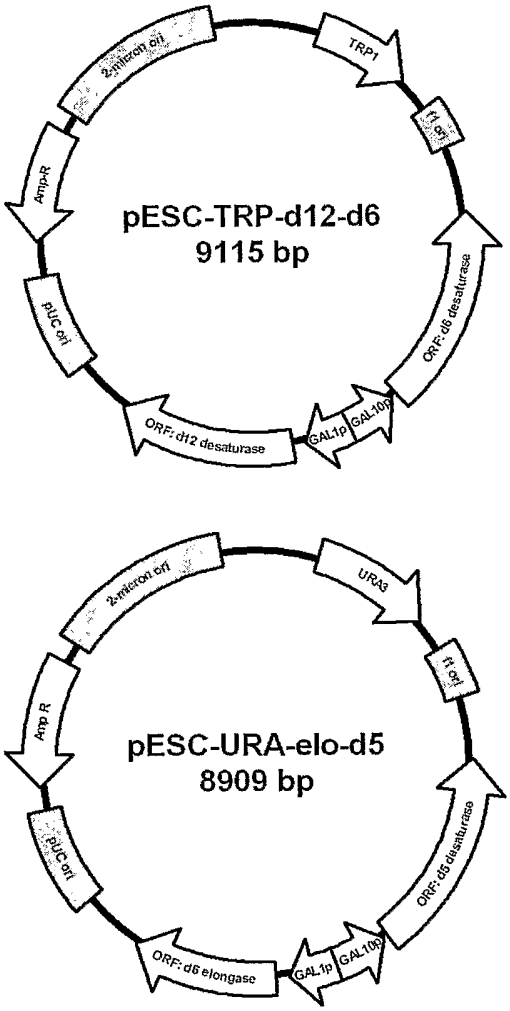

pESC-TRP-d12-d6
9115 bp
2-micron ori
TRP1
f1 ori
ORF: d6 desaturase
GAL10p
GAL1p
ORF: d12 desaturase
pUC ori
Amp R
pESC-URA-elo-d5
8909 bp
2-micron ori
URA3
f1 ori
ORF: d5 desaturase
GAL10p
GAL1p
ORF: d6 elongase
pUC ori
Amp R

Fig. 9

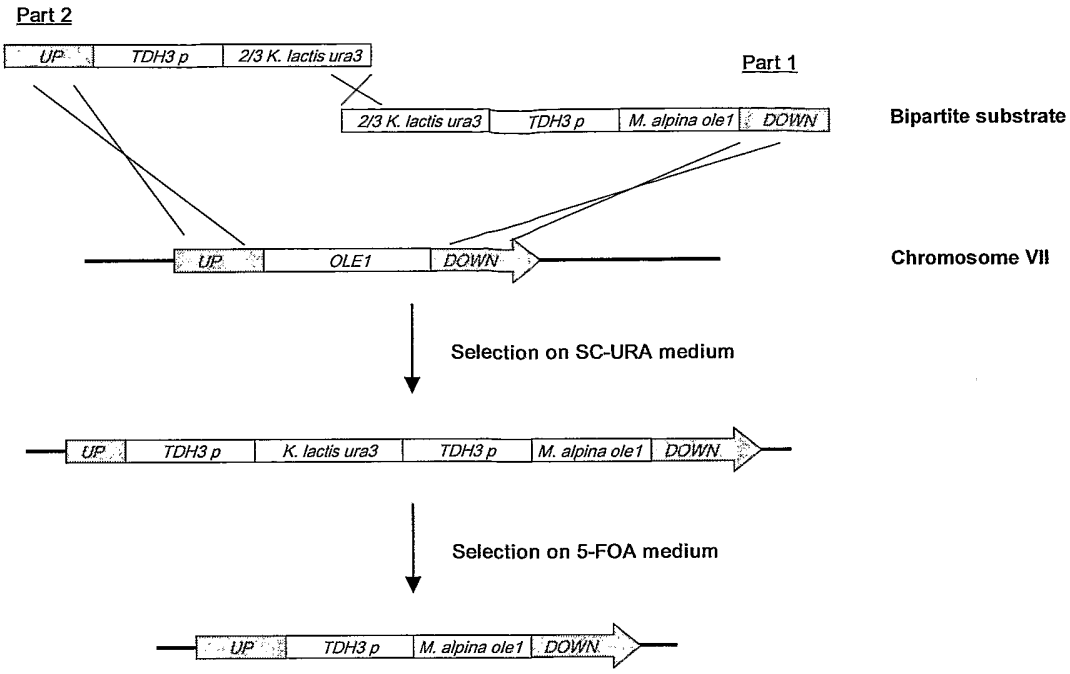

Part 2
UP
TDH3 p
2/3 K. lactis ura3
Part 1
2/3 K. lactis ura3
TDH3 p
M. alpina ole1
DOWN
Bipartite substrate
UP
OLE1
DOWN
Chromosome VII
Selection on SC-URA medium
UP
TDH3 p
K. lactis ura3
TDH3 p
M. alpina ole1
DOWN
Selection on 5-FOA medium
UP
TDH3 p
M. alpina ole1
DOWN

Fig. 10

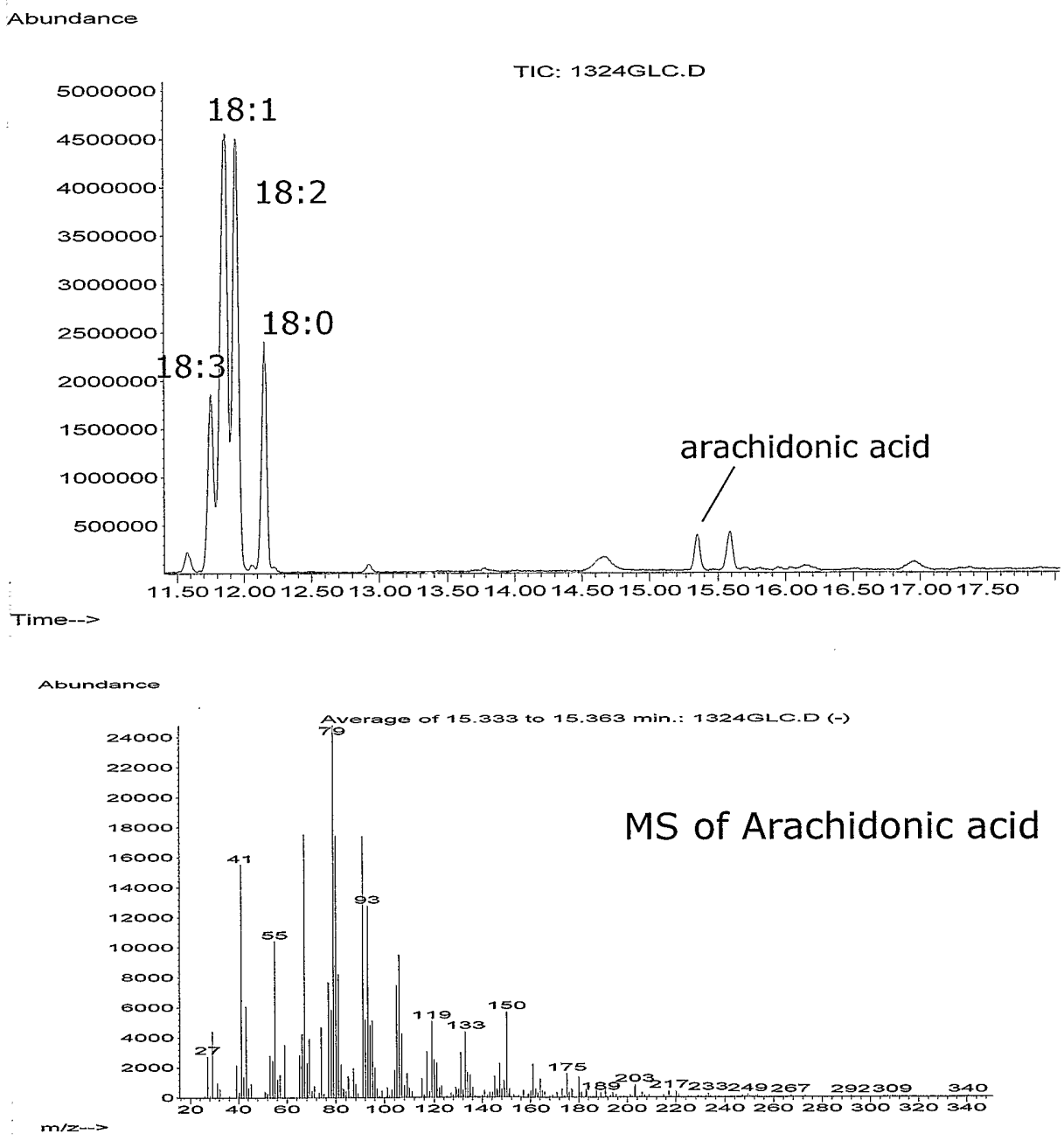

Abundance
TIC: 1324GLC.D
18:1
18:2
18:0
18:3
arachidonic acid
Time-->
Abundance
Average of 15.333 to 15.363 min.: 1324GLC.D (-)
MS of Arachidonic acid
m/z-->

Fig. 11

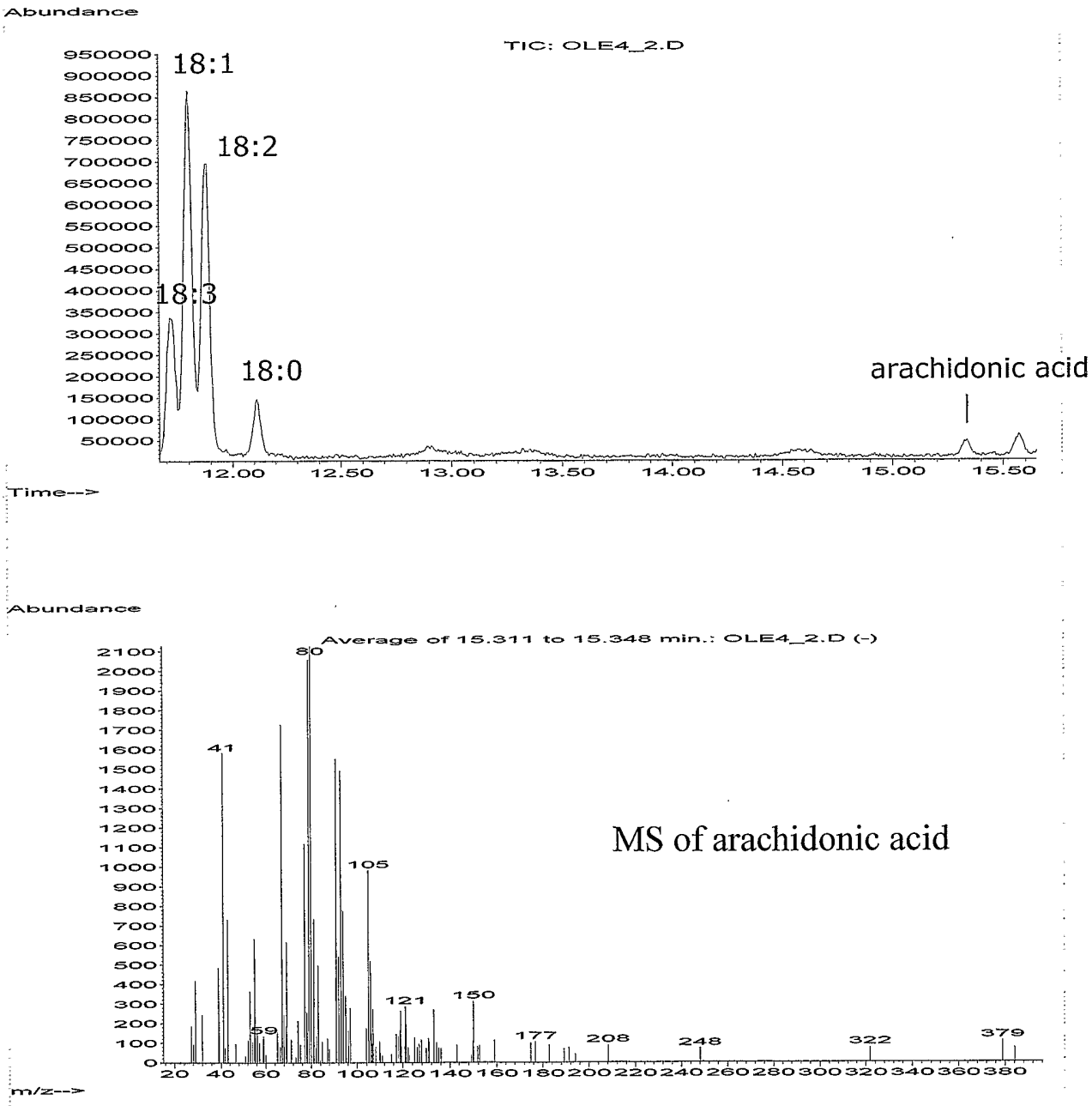

Abundance
TIC: OLE4_2.D
18:1
18:2
18:3
18:0
arachidonic acid
Time-->
Abundance
Average of 15.311 to 15.348 min.: OLE4_2.D (-)
MS of arachidonic acid
m/z-->

Fig. 12

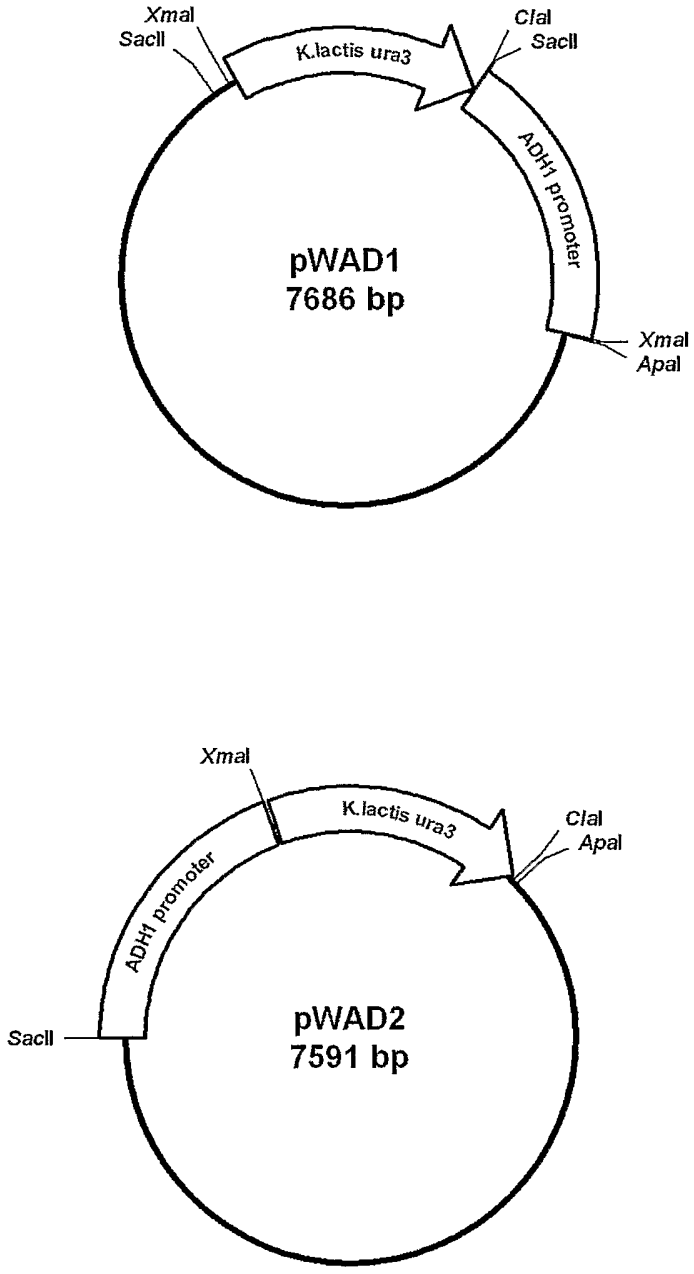
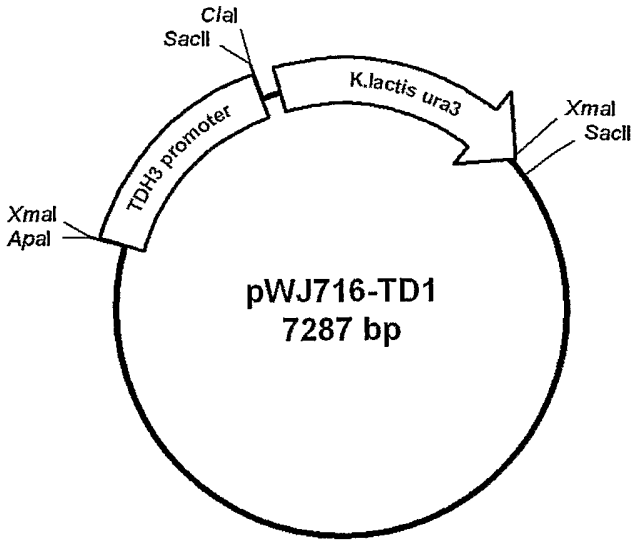

XmaI
SacII
K.lactis ura3
ClaI
SacII
ADH1 promoter
pWAD1
7686 bp
XmaI
ApaI
XmaI
K.lactis ura3
ClaI
ApaI
ADH1 promoter
SacII
pWAD2
7591 bp

Fig. 13


ClaI
SacII
K.lactis ura3
XmaI
SacII
TDH3 promoter
XmaI
ApaI
pWJ716-TD1
7287 bp

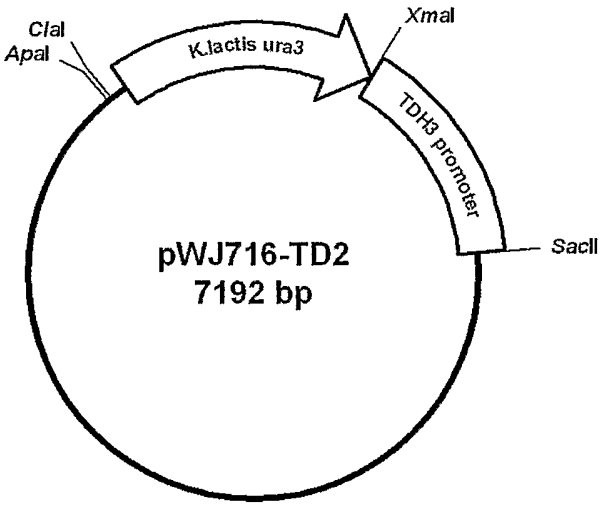

ClaI
ApaI
K.lactis ura3
XmaI
TDH3 promoter
SacII
pWJ716-TD2
7192 bp

Fig. 14

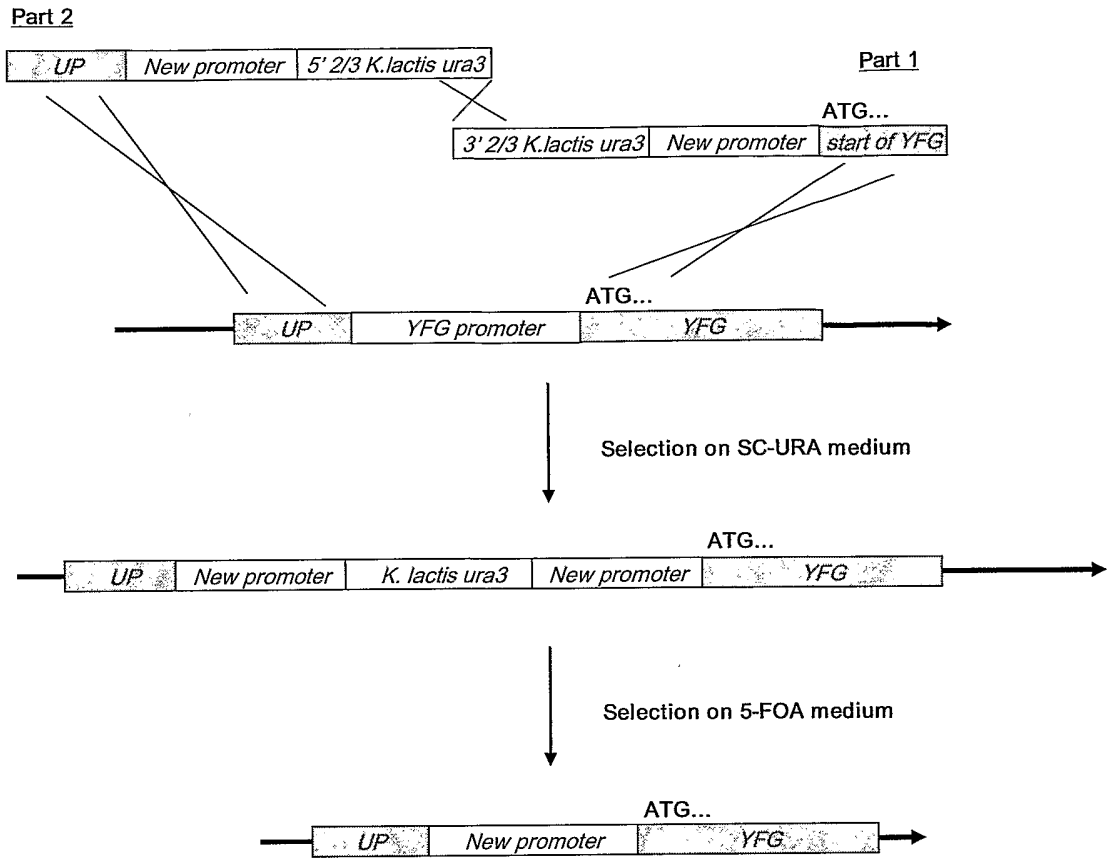

Part 2
UP
New promoter
5' 2/3 K.lactis ura3
Part 1
ATG...
3' 2/3 K.lactis ura3
New promoter
start of YFG
ATG...
UP
YFG promoter
YFG
Selection on SC-URA medium
ATG...
UP
New promoter
K. lactis ura3
New promoter
YFG
Selection on 5-FOA medium
ATG...
UP
New promoter
YFG

Fig. 15

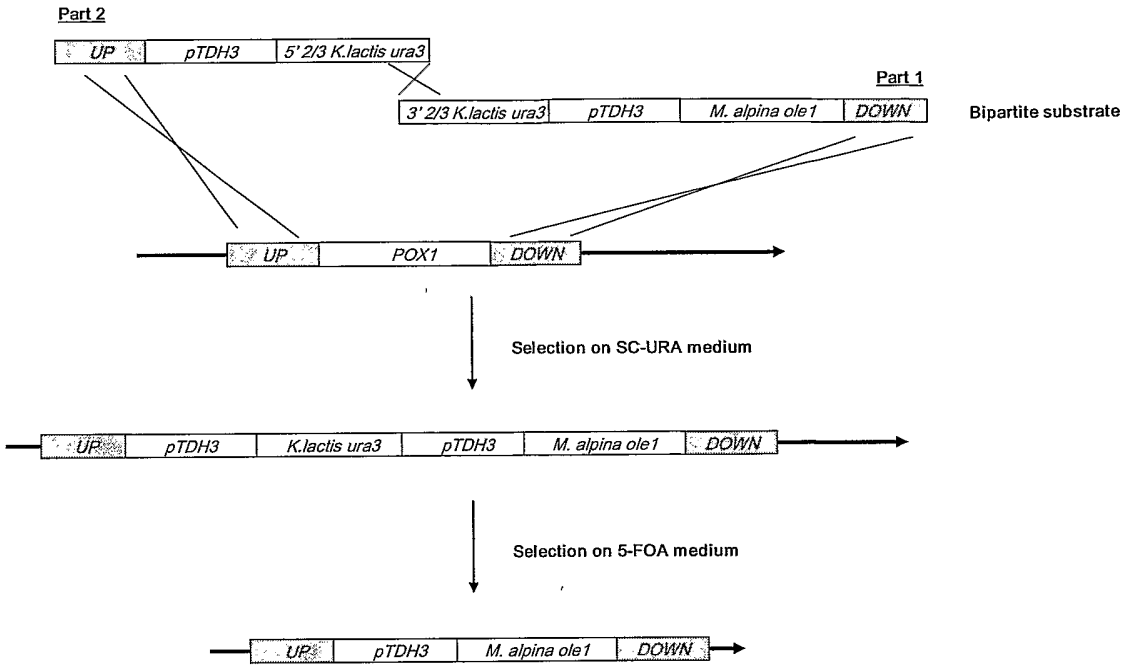

Part 2
UP
pTDH3
5' 2/3 K.lactis ura3
Part 1
3' 2/3 K.lactis ura3
pTDH3
M. alpina ole1
DOWN
Bipartite substrate
UP
POX1
DOWN
Selection on SC-URA medium
UP
pTDH3
K.lactis ura3
pTDH3
M. alpina ole1
DOWN
Selection on 5-FOA medium
UP
pTDH3
M. alpina ole1
DOWN

Fig. 16

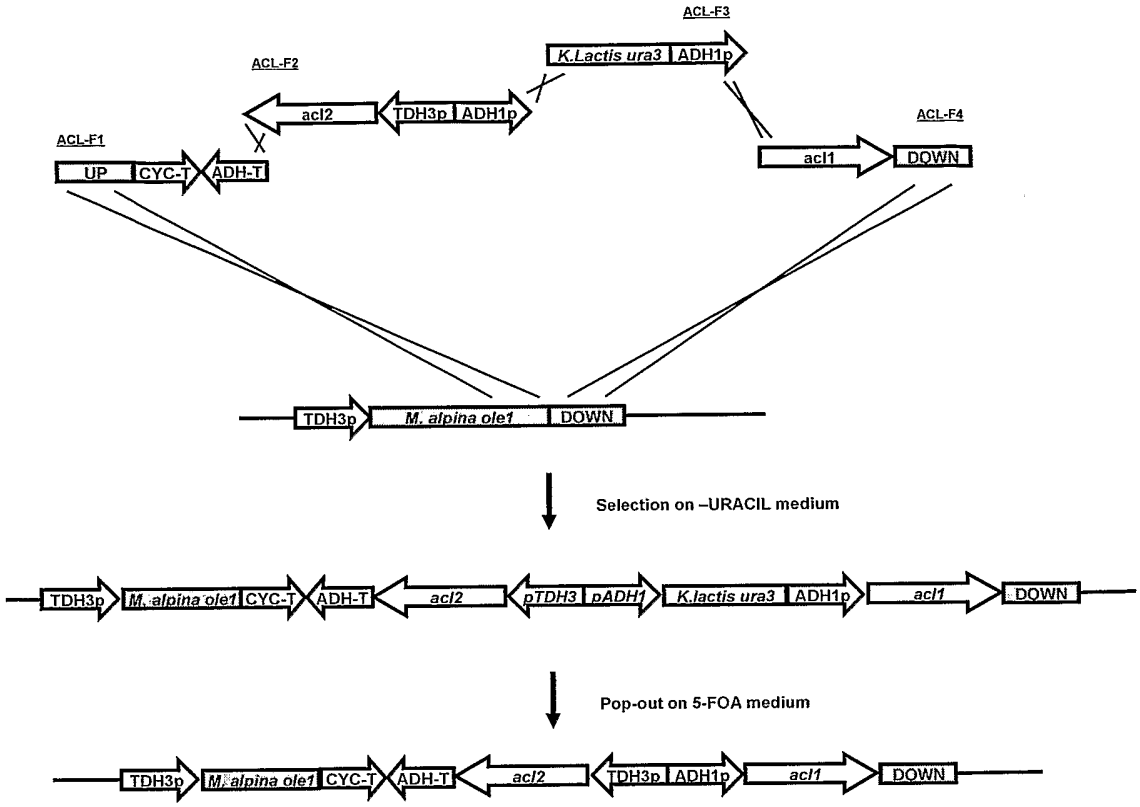

ACL-F3
K.Lactis ura3
ADH1p
ACL-F2
acl2
TDH3p
ADH1p
ACL-F4
ACL-F1
UP
CYC-T
ADH-T
acl1
DOWN
TDH3p
M. alpina ole1
DOWN
Selection on –URACIL medium
TDH3p
M. alpina ole1
CYC-T
ADH-T
acl2
pTDH3
pADH1
K.lactis ura3
ADH1p
acl1
DOWN
Pop-out on 5-FOA medium
TDH3p
M. alpina ole1
CYC-T
ADH-T
acl2
TDH3p
ADH1p
acl1
DOWN

Fig. 17

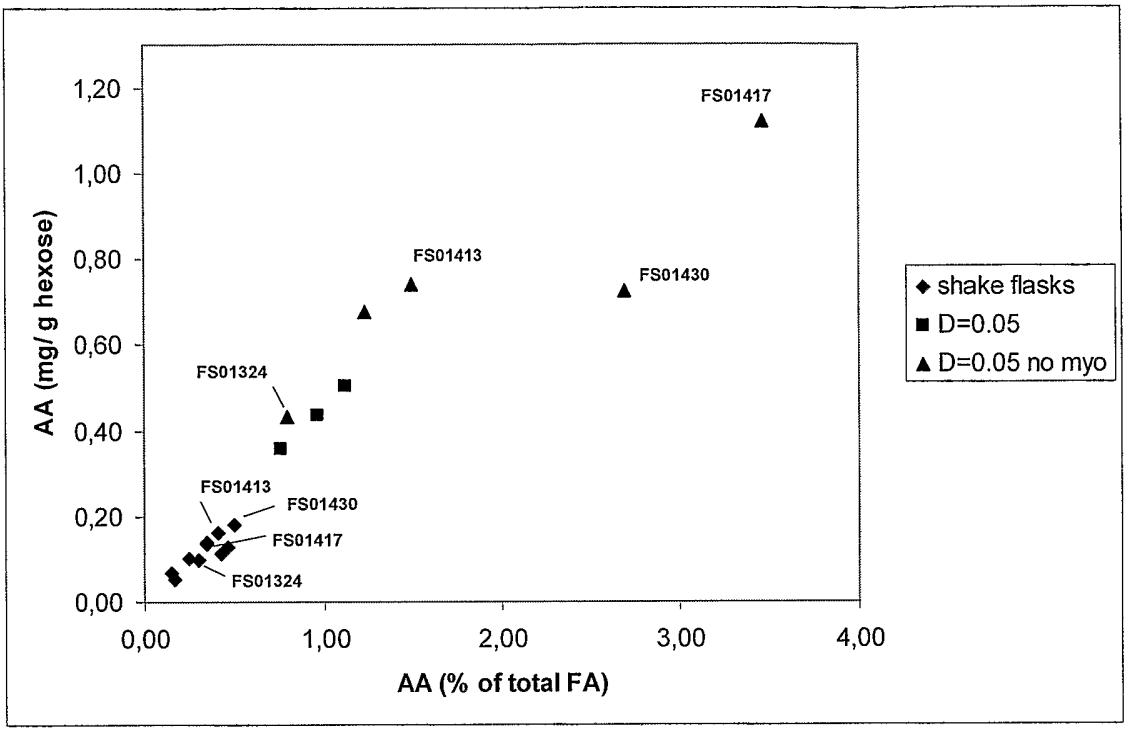

1,20
1,00
0,80
0,60
0,40
0,20
0,00
AA (mg/ g hexose)
0,00
1,00
2,00
3,00
4,00
AA (% of total FA)
FS01417
FS01413
FS01430
FS01324
FS01413
FS01430
FS01417
FS01324
shake flasks
D=0.05
D=0.05 no myo

Fig. 18

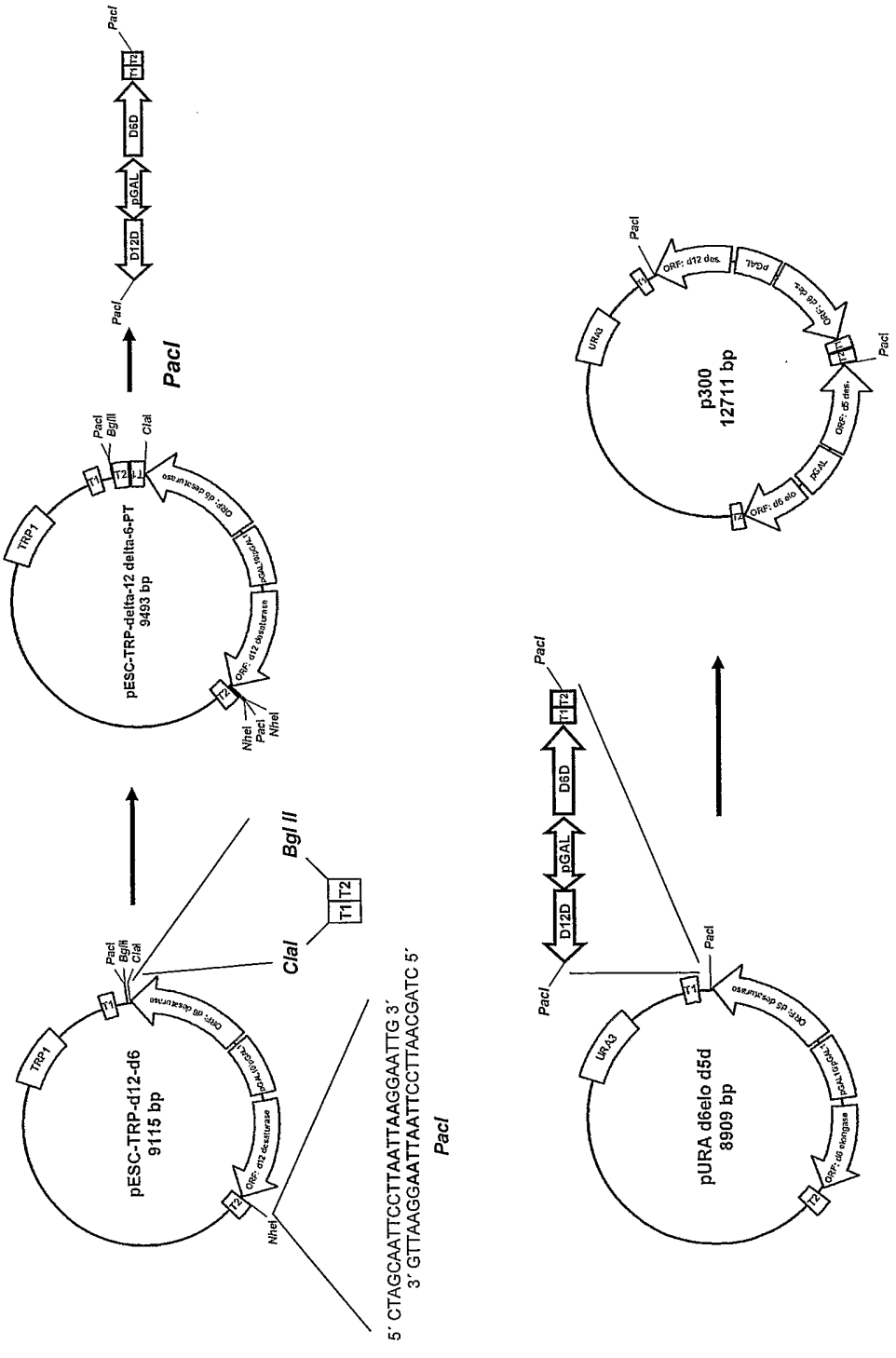
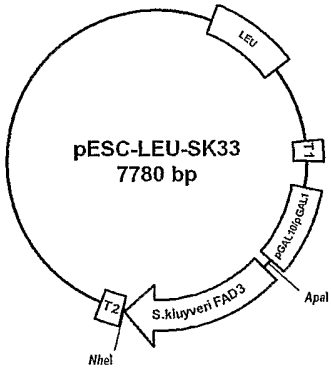
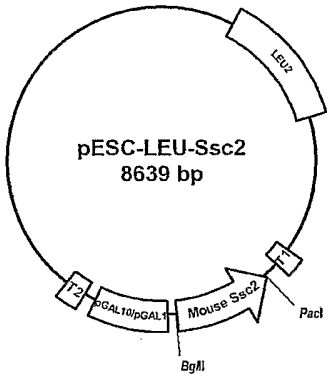

pESC-TRP-d12-d6
9115 bp
pESC-TRP-delta-12 delta-6-PT
9493 bp
p300
12711 bp
pURA d6elo d5d
8909 bp
PacI
ClaI
Bgl II
NheI
5´ CTAGCAATTCCTTAATTAAGGAATTG 3´
3´ GTTAAGGAATTAATTCCTTAACGATC 5´
PacI

Fig. 19


LEU
pESC-LEU-SK33
7780 bp
T1
pGAL10/pGAL1
ApaI
T2
S.kluyveri FAD3
NheI


LEU2
pESC-LEU-Ssc2
8639 bp
T1
T2
pGAL10/pGAL1
Mouse Ssc2
PacI
BglII

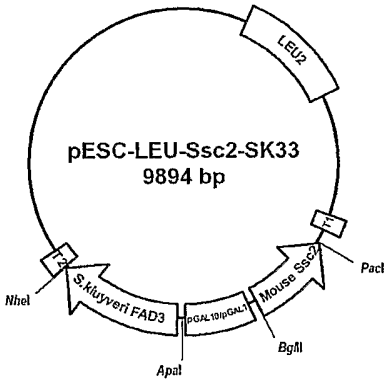
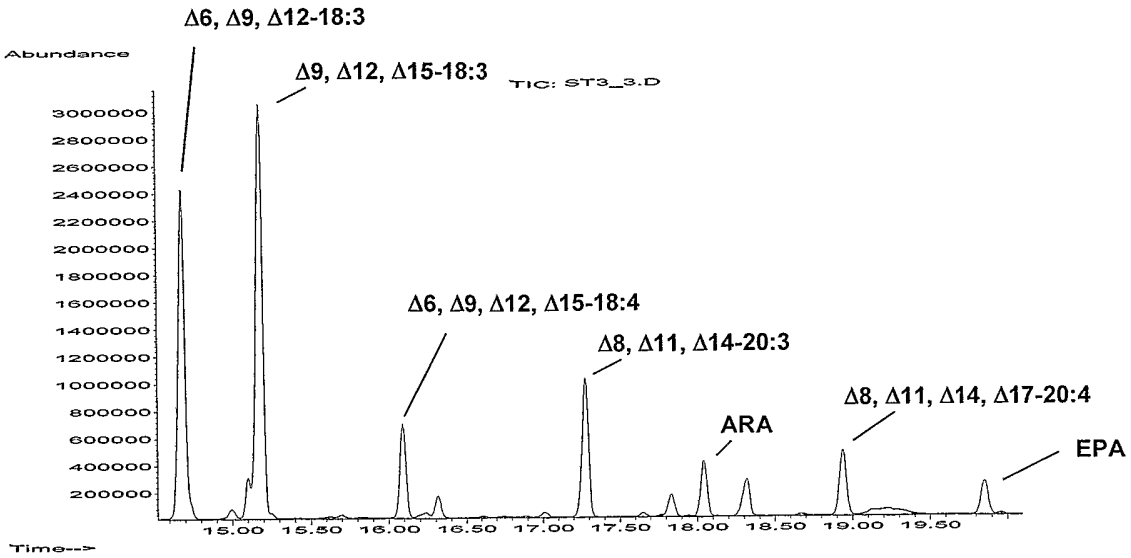
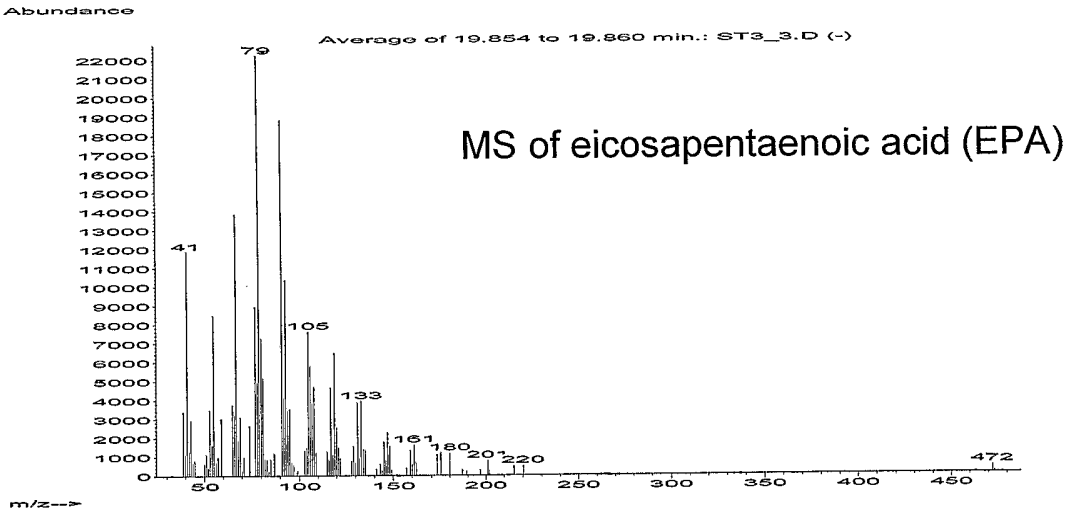

LEU2
pESC-LEU-Ssc2-SK33
9894 bp
T1
PacI
T2
NheI
S.kluyveri FAD3
pGAL10/pGAL1
Mouse Ssc2
BglII
ApaI

Fig. 20





MS of eicosapentaenoic acid (EPA)

Fig. 21

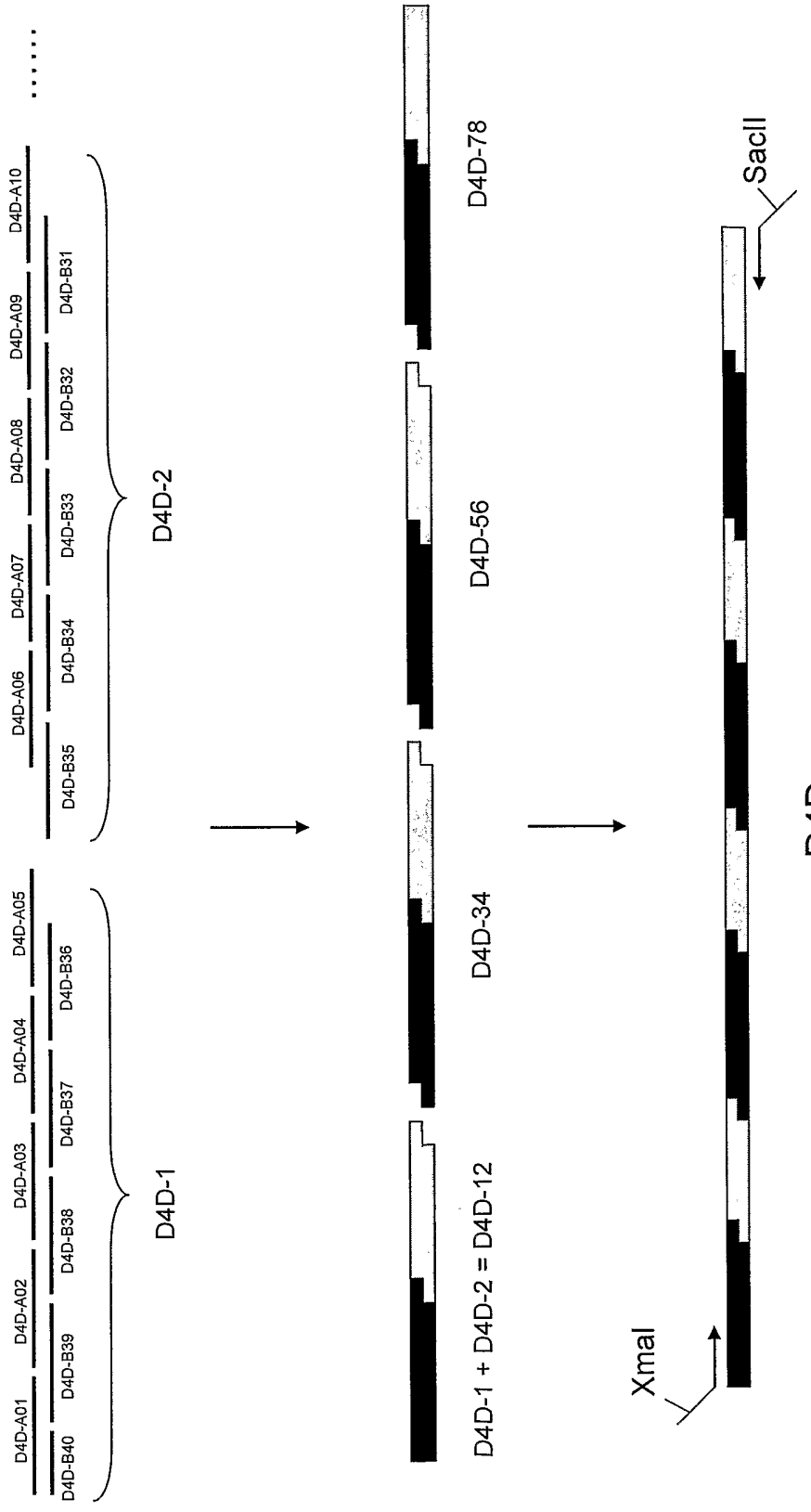

D4D-A01
D4D-A02
D4D-A03
D4D-A04
D4D-A05
D4D-B40
D4D-B39
D4D-B38
D4D-B37
D4D-B36
D4D-1
D4D-A06
D4D-A07
D4D-A08
D4D-A09
D4D-A10
D4D-B35
D4D-B34
D4D-B33
D4D-B32
D4D-B31
D4D-2
.....
D4D-1 + D4D-2 = D4D-12
D4D-34
D4D-56
D4D-78
XmaI
SacII
D4D

Fig. 22

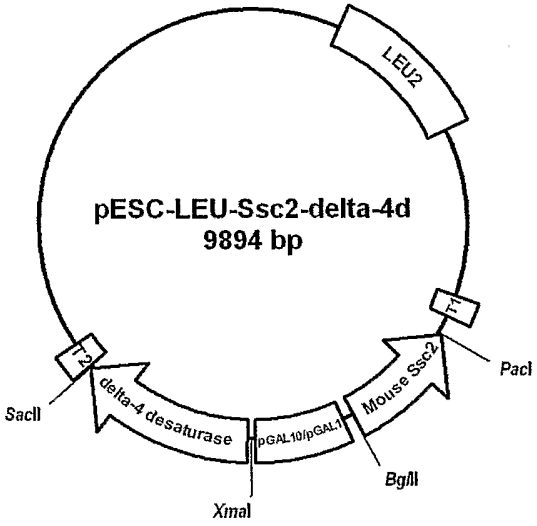

LEU2
pESC-LEU-Ssc2-delta-4d
9894 bp
T-1
T-2
SacII
delta-4 desaturase
pGAL10/pGAL1
Mouse Ssc2
PacI
XmaI
BglII

Fig. 23

24/24

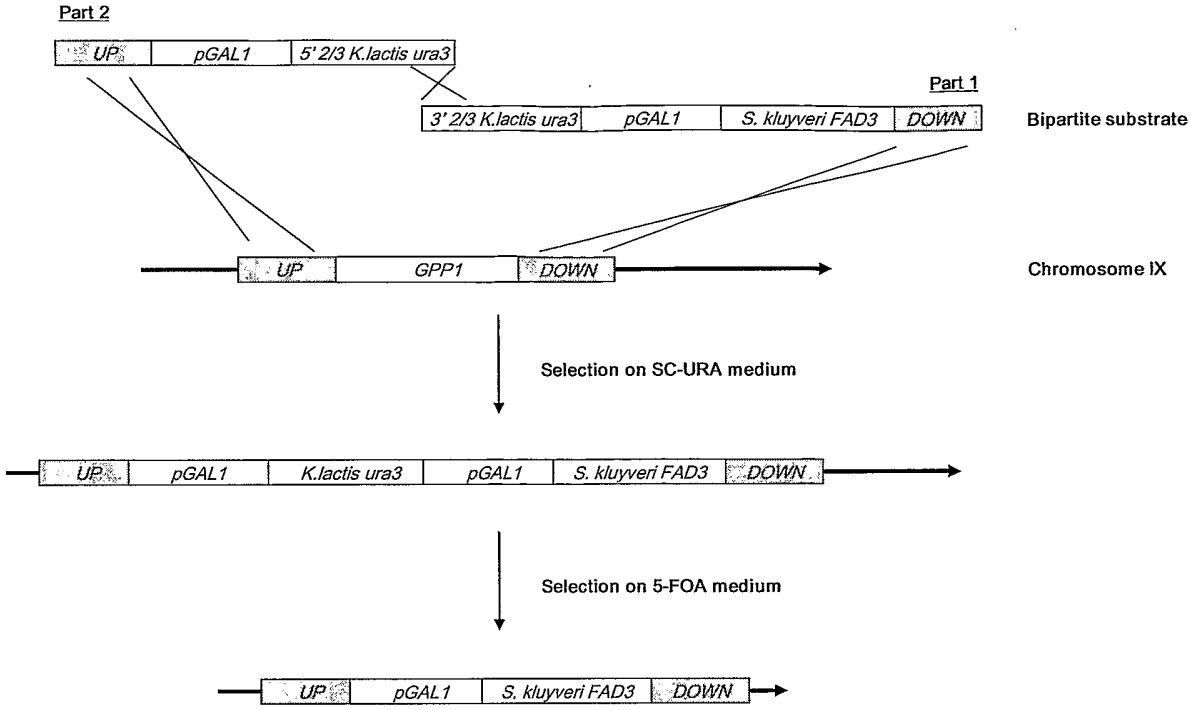

Part 2
UP
pGAL1
5' 2/3 K.lactis ura3
Part 1
3' 2/3 K.lactis ura3
pGAL1
S. kluyveri FAD3
DOWN
Bipartite substrate
UP
GPP1
DOWN
Chromosome IX
Selection on SC-URA medium
UP
pGAL1
K.lactis ura3
pGAL1
S. kluyveri FAD3
DOWN
Selection on 5-FOA medium
UP
pGAL1
S. kluyveri FAD3
DOWN

Fig. 24

SEQUENCE LISTING

<110> Fluxome Sciences
Jochen Förster
Nina Katarina Gunnarsson
Jens Bredal Nielsen

<120> METABOLICALLY ENGINEERED CELLS FOR THE PRODUCTION OF POLYUNSATURATED FATTY ACIDS

<130> P36601PC01

<150> 60/577,245
<151> 2004-06-04

<160> 114

<170> FastSEQ for Windows Version 4.0

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<211> 1338
<212> DNA
<213> Mortierella alpina

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gatcctctcc agcacgagga actgcccccc ctcttccccg agaaaatcac cgtctacaac 120
atctggagat atcttgacta caagcatgtt ttcggtctgg ggctgacgcc tttgatcgca 180
ctctacggtc tcttgacgac cgagatccag acgaagacgc tgatctggtc catcatctac 240
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755 760 765
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785 790 795 800
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865 870 875 880
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945 950 955 960
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1250

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<213> Mortierella alpina

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<211> 1687
<212> DNA
<213> Borago officinalis

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<212> DNA
<213> Anemone levellei

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<211> 1332

<212> DNA
<213> Caenorhabditis elegans

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<211> 957
<212> DNA
<213> Mortierella alpina

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<210> 17
<211> 1192
<212> DNA
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<211> 867
<212> DNA
<213> Caenorhabditis elegans

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<211> 900
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<213> Mus musculus

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<213> Thraustochytrium aureum

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<212> DNA
<213> Phytophthora infestans

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<212> DNA
<213> Mortierella alpina

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<213> Phytophthora megasperma

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<213> Thraustochytrium sp. ATCC 21685

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<213> Caenorhabditis elegans

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<213> Phaeodactylum tricornutum

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<210> 29
<211> 900
<212> DNA
<213> Homo sapiens

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<212> DNA
<213> Caenorhabditis elegans

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ctatcgctct cagtataaga aggaggccat tggcctcgcc ctgcactgga ccttgaaggc 840
cctgttccac ttattcttta tgcccagcat cctcacatcg ctgttggtgt ttttcgtttc 900
ggagctggtt ggcggcttcg gcattgcgat cgtggtgttc atgaaccact acccactgga 960
gaagatcggg gacccagtct gggatggcca tggattctcg gttggccaga tccatgagac 1020
catgaacatt cggcgaggga ttatcacaga ttggtttttc ggaggcttga attaccagat 1080
tgagcaccat ttgtggccga ccctccctcg ccacaacctg acagcggtta gctaccaggt 1140

ggaacagctg tgccagaagc acaacctgcc gtatcggaac ccgctgcccc atgaagggtt 1200
ggtcatcctg ctgcgctatc tggcggtgtt cgcccggatg gcggagaagc aacccgcggg 1260
gaaggctcta taagg 1275

<210> 39
<211> 445
<212> PRT
<213> Mortierella alpina

<400> 39

```
Met Ala Thr Pro Leu Pro Pro Ser Phe Val Val Pro Ala Thr Gln Thr
 1               5                  10                  15
Glu Thr Arg Arg Asp Pro Leu Gln His Glu Glu Leu Pro Pro Leu Phe
            20                  25                  30
Pro Glu Lys Ile Thr Val Tyr Asn Ile Trp Arg Tyr Leu Asp Tyr Lys
        35                  40                  45
His Val Phe Gly Leu Gly Leu Thr Pro Leu Ile Ala Leu Tyr Gly Leu
    50                  55                  60
Leu Thr Thr Glu Ile Gln Thr Lys Thr Leu Ile Trp Ser Ile Ile Tyr
65                  70                  75                  80
Tyr Tyr Ala Thr Gly Leu Gly Ile Thr Ala Gly Tyr His Arg Leu Trp
                85                  90                  95
Ala His Arg Ala Tyr Asn Ala Gly Pro Ala Met Ser Phe Val Leu Ala
            100                 105                 110
Leu Leu Gly Ala Gly Ala Val Glu Gly Ser Ile Lys Trp Trp Ser Arg
        115                 120                 125
Gly His Arg Ala His His Arg Trp Thr Asp Thr Glu Lys Asp Pro Tyr
    130                 135                 140
Ser Ala His Arg Gly Leu Phe Phe Ser His Ile Gly Trp Met Leu Ile
145                 150                 155                 160
Lys Arg Pro Gly Trp Lys Ile Gly His Ala Asp Val Asp Asp Leu Asn
                165                 170                 175
Lys Ser Lys Leu Val Gln Trp Gln His Lys Asn Tyr Leu Pro Leu Val
            180                 185                 190
Leu Ile Met Gly Val Val Phe Pro Thr Val Val Ala Gly Leu Gly Trp
        195                 200                 205
Gly Asp Trp Arg Gly Gly Tyr Phe Phe Ala Ala Ile Leu Arg Leu Val
    210                 215                 220
Phe Val His His Ala Thr Phe Cys Val Asn Ser Leu Ala His Trp Leu
225                 230                 235                 240
Gly Asp Gly Pro Phe Asp Asp Arg His Ser Pro Arg Asp His Phe Ile
                245                 250                 255
Thr Ala Phe Val Thr Leu Gly Glu Gly Tyr His Asn Phe His His Gln
            260                 265                 270
Phe Pro Gln Asp Tyr Arg Asn Ala Ile Arg Phe Tyr Gln Tyr Asp Pro
        275                 280                 285
Thr Lys Trp Val Ile Ala Leu Cys Ala Phe Phe Gly Leu Ala Thr His
    290                 295                 300
Leu Lys Thr Phe Pro Glu Asn Glu Val Arg Lys Gly Gln Leu Gln Met
305                 310                 315                 320
Ile Glu Lys Arg Val Leu Glu Lys Lys Thr Lys Leu Gln Trp Gly Thr
                325                 330                 335
Pro Ile Ala Asp Leu Pro Ile Leu Ser Phe Glu Asp Phe Gln His Ala
            340                 345                 350
Cys Lys Asn Asp Asn Lys Lys Trp Ile Leu Leu Glu Gly Val Val Tyr
        355                 360                 365
Asp Val Ala Asp Phe Met Thr Glu His Pro Gly Gly Glu Lys Tyr Ile
    370                 375                 380
Lys Met Gly Val Gly Lys Asp Met Thr Ala Ala Phe Asn Gly Gly Met
```

385 390 395 400
Tyr Asp His Ser Asn Ala Ala Arg Asn Leu Leu Ser Leu Met Arg Val
405 410 415
Ala Val Val Glu Phe Gly Gly Glu Val Glu Ala Gln Lys Lys Asn Pro
420 425 430
Ser Met Pro Ile Tyr Gly Thr Asp His Val Lys Ala Glu
435 440 445

<210> 40
<211> 493
<212> PRT
<213> Cryptococcus curvatus

<400> 40
Met Ser Ala Ser Thr Ala Thr Ala Pro Pro Ala Thr Ala Pro Ala Val
1 5 10 15
Ala Asn Pro Thr Pro Ala Ala Ala Ser Ala Ala Ala Ala Ala Pro Ala
20 25 30
Ala Thr Lys Asp Lys Ala Glu Thr Ile Asp Pro Glu Ser Glu His Phe
35 40 45
Val Val Ser Gln Asn Tyr Val Thr Arg Thr Val Glu Asn Met Thr Met
50 55 60
Leu Pro Pro Val Thr Trp Ser Asn Leu Leu Gln Asn Ile Gln Trp Ile
65 70 75 80
Ser Phe Thr Ala Leu Thr Val Pro Pro Ala Met Ala Ile Tyr Gly Leu
85 90 95
Cys Thr Leu Glu Leu Gln Arg Lys Thr Val Ile Trp Ala Ile Val Tyr
100 105 110
Tyr Phe Met Thr Gly Leu Gly Ile Thr Ala Gly Tyr His Arg Leu Trp
115 120 125
Ala His Arg Ala Tyr Asn Ala Ser Ala Pro Leu Gln Tyr Phe Leu Ala
130 135 140
Leu Cys Gly Ala Gly Ser Val Gln Gly Ser Ile Lys Trp Trp Ser Arg
145 150 155 160
Gly His Arg Ala His His Arg Tyr Thr Asp Thr Lys Leu Asp Pro Tyr
165 170 175
Ser Ala His Glu Gly Phe Trp Trp Ala His Val Gly Trp Met Leu Val
180 185 190
Lys Pro Arg Gly Lys Ile Gly Val Ala Asp Ile Ser Asp Leu Ser Arg
195 200 205
Asn Pro Val Val Lys Trp Gln His Asn Asn Tyr Val Met Leu Met Val
210 215 220
Leu Met Gly Leu Val Phe Pro Thr Leu Val Ala Gly Leu Gly Trp Gly
225 230 235 240
Asp Trp Lys Gly Gly Leu Leu Phe Ala Gly Ala Ala Arg Leu Val Phe
245 250 255
Val His His Ser Thr Phe Cys Val Asn Ser Leu Ala His Trp Leu Gly
260 265 270
Glu Thr Pro Phe Asp Asn Lys His Thr Pro Lys Asp His Phe Ile Thr
275 280 285
Ala Leu Val Thr Val Gly Glu Gly Tyr His Asn Phe His His Gln Phe
290 295 300
Pro Met Asp Phe Arg Asn Ala Ile Lys Trp Tyr Gln Tyr Asp Pro Thr
305 310 315 320
Lys Trp Phe Ile Trp Thr Met Ser Asn Val Gly Leu Ala Ser His Leu
325 330 335
Lys Lys Phe Pro Asp Asn Glu Ile Lys Lys Gly Gln Tyr Thr Met Lys
340 345 350

Leu Gln Met Leu Gln Glu Gln Ser Gly Ser Ile Gln Trp Pro Lys His
355 360 365
Ser Asn Asp Leu Pro Val Ile Ser Trp Glu Asp Phe Gln Ala Glu Ala
370 375 380
Lys Glu Arg Ser Leu Val Ala Ile His Gly Phe Ile His Asp Cys Ser
385 390 395 400
Ser Phe Leu Glu Asp His Pro Gly Gly Ile His Leu Ile Lys Lys Ala
405 410 415
Ile Gly Thr Asp Ala Thr Thr Ala Phe Phe Gly Gly Val Tyr Asp His
420 425 430
Ser Asn Ala Ala His Asn Leu Leu Ala Met Met Arg Val Gly Ile Leu
435 440 445
Asp Gly Gly Met Glu Val Glu Ser Leu Lys Leu Glu Asn Leu Gln Arg
450 455 460
Ser Met Ser Val Ser Ser Met Glu Ser Asp Ala Ala Ser Ser Ala Ser
465 470 475 480
Ser Val Ser Val Ser Ser Ile Ser Pro Arg Trp Pro His
485 490

<210> 41
<211> 476
<212> PRT
<213> Histoplasma capsulatus

<400> 41
Met Ala Leu Asn Glu Ala Pro Thr Ala Ser Pro Val Ala Glu Thr Ala
1 5 10 15
Ala Gly Gly Lys Asp Val Val Thr Asp Ala Ala Arg Arg Pro Asn Ser
20 25 30
Glu Pro Lys Lys Val His Ile Thr Asp Thr Pro Ile Thr Leu Ala Asn
35 40 45
Trp His Lys His Ile Ser Trp Leu Asn Val Thr Leu Ile Ile Ala Ile
50 55 60
Pro Ile Tyr Gly Leu Val Gln Ala Tyr Trp Val Pro Leu His Leu Lys
65 70 75 80
Thr Ala Leu Trp Ala Val Val Tyr Tyr Phe Met Thr Gly Leu Gly Ile
85 90 95
Thr Ala Gly Tyr His Arg Leu Trp Ala His Cys Ser Tyr Ser Ala Thr
100 105 110
Leu Pro Leu Lys Ile Tyr Leu Ala Ala Val Gly Gly Gly Ala Val Glu
115 120 125
Gly Ser Ile Arg Trp Trp Ala Arg Gly His Arg Ala His His Arg Tyr
130 135 140
Thr Asp Thr Asp Lys Asp Pro Tyr Ser Val Arg Lys Gly Leu Leu Tyr
145 150 155 160
Ser His Ile Gly Trp Met Val Met Lys Gln Asn Pro Lys Arg Ile Gly
165 170 175
Arg Thr Glu Ile Thr Asp Leu Asn Glu Asp Pro Val Val Val Trp Gln
180 185 190
His Arg Asn Tyr Leu Lys Val Val Ile Phe Met Gly Ile Val Phe Pro
195 200 205
Met Leu Val Ser Gly Leu Gly Trp Gly Asp Trp Phe Gly Gly Phe Ile
210 215 220
Tyr Ala Gly Ile Leu Arg Ile Phe Phe Val Gln Gln Ala Thr Phe Cys
225 230 235 240
Val Asn Ser Leu Ala His Trp Leu Gly Asp Gln Pro Phe Asp Asp Arg
245 250 255
Asn Ser Pro Arg Asp His Ile Val Thr Ala Leu Val Thr Leu Gly Glu

```
                260                 265                 270
Gly Tyr His Asn Phe His His Glu Phe Pro Ser Asp Tyr Arg Asn Ala
        275                 280                 285
Ile Glu Trp His Gln Tyr Asp Pro Thr Lys Trp Thr Ile Trp Ile Trp
    290                 295                 300
Lys Gln Leu Gly Leu Ala Tyr Asp Leu Lys Gln Phe Arg Ala Asn Glu
305                 310                 315                 320
Ile Glu Lys Gly Arg Val Gln Gln Leu Gln Lys Lys Ile Asp Gln Arg
                325                 330                 335
Arg Ala Lys Leu Asp Trp Gly Ile Pro Leu Glu Gln Leu Pro Val Ile
            340                 345                 350
Glu Trp Asp Asp Tyr Val Asp Gln Ala Lys Asn Gly Arg Gly Leu Ile
        355                 360                 365
Ala Ile Ala Gly Val Val His Asp Val Thr Asp Phe Ile Lys Asp His
    370                 375                 380
Pro Gly Gly Lys Ala Met Ile Asn Ser Gly Ile Gly Lys Asp Ala Thr
385                 390                 395                 400
Ala Met Phe Asn Gly Gly Val Tyr Asn His Ser Asn Ala Ala His Asn
                405                 410                 415
Gln Leu Ser Thr Met Arg Val Gly Val Ile Arg Gly Gly Cys Glu Val
            420                 425                 430
Glu Ile Trp Lys Arg Ala Gln Lys Glu Asn Lys Glu Val Glu Ser Val
        435                 440                 445
Arg Asp Glu Tyr Gly Asn Arg Ile Val Arg Ala Gly Ala Gln Val Thr
    450                 455                 460
Lys Ile Pro Glu Pro Ile Thr Thr Ala Asp Ala Ala
465                 470                 475
```

<210> 42
<211> 353
<212> PRT
<213> Trichoplusia ni

<400> 42

```
Met Pro Pro Gln Gly Gln Thr Gly Gly Ser Trp Val Leu Tyr Glu Thr
 1               5                  10                  15
Asp Ala Val Asn Thr Asp Thr Asp Ala Pro Val Ile Val Pro Pro Ser
            20                  25                  30
Ala Glu Lys Arg Glu Trp Lys Ile Val Trp Arg Asn Val Ile Leu Met
        35                  40                  45
Gly Met Leu His Ile Gly Gly Val Tyr Gly Ala Tyr Leu Phe Leu Thr
    50                  55                  60
Lys Ala Met Trp Leu Thr Asp Leu Phe Ala Phe Phe Leu Tyr Leu Cys
65                  70                  75                  80
Ser Gly Leu Gly Ile Thr Ala Gly Ala His Arg Leu Trp Ala His Lys
                85                  90                  95
Ser Tyr Lys Ala Arg Leu Pro Leu Arg Leu Leu Leu Thr Leu Phe Asn
            100                 105                 110
Thr Leu Ala Phe Gln Asp Ala Val Ile Asp Trp Ala Arg Asp His Arg
        115                 120                 125
Met His His Lys Tyr Ser Glu Thr Asp Ala Asp Pro His Asn Ala Thr
    130                 135                 140
Arg Gly Phe Phe Phe Ser His Val Gly Trp Leu Leu Val Arg Lys His
145                 150                 155                 160
Pro Gln Ile Lys Ala Lys Gly His Thr Ile Asp Leu Ser Asp Leu Lys
                165                 170                 175
Ser Asp Pro Ile Leu Arg Phe Gln Lys Lys Tyr Tyr Leu Thr Leu Met
            180                 185                 190
```

Pro Leu Ile Cys Phe Ile Leu Pro Ser Tyr Ile Pro Thr Leu Trp Gly
195 200 205
Glu Ser Ala Phe Asn Ala Phe Phe Val Cys Ser Ile Phe Arg Tyr Val
210 215 220
Tyr Val Leu Asn Val Thr Trp Leu Val Asn Ser Ala Ala His Leu Trp
225 230 235 240
Gly Ser Lys Pro Tyr Asp Lys Asn Ile Asn Pro Val Glu Thr Arg Pro
245 250 255
Val Ser Leu Val Val Leu Gly Glu Gly Phe His Asn Tyr His His Thr
260 265 270
Phe Pro Trp Asp Tyr Lys Thr Ala Glu Leu Gly Asp Tyr Ser Leu Asn
275 280 285
Phe Thr Lys Met Phe Ile Asp Phe Met Ala Ser Ile Gly Trp Ala Tyr
290 295 300
Asp Leu Lys Thr Val Ser Thr Asp Val Ile Gln Lys Arg Val Lys Arg
305 310 315 320
Thr Gly Asp Gly Ser His Ala Val Trp Gly Trp Asp Asp His Glu Val
325 330 335
His Gln Glu Asp Lys Lys Leu Ala Ala Ile Ile Asn Pro Glu Lys Thr
340 345 350
Glu

<210> 43
<211> 401
<212> PRT
<213> Mortierella alpina

<400> 43
Met Ala Pro Pro Asn Thr Ile Asp Ala Gly Leu Thr Gln Arg His Ile
1 5 10 15
Ser Thr Ser Ala Ala Pro Asn Ser Ala Lys Pro Thr Phe Glu Arg Asn
20 25 30
Tyr Gln Leu Pro Glu Phe Thr Ile Lys Glu Ile Arg Glu Cys Ile Pro
35 40 45
Ala His Cys Phe Glu Arg Ser Gly Leu Arg Gly Leu Cys His Val Ala
50 55 60
Ile Asp Leu Thr Trp Ala Ser Leu Leu Phe Leu Ala Ala Thr Gln Ile
65 70 75 80
Asp Lys Phe Glu Asn Pro Leu Ile Arg Tyr Leu Ala Trp Pro Val Tyr
85 90 95
Trp Ile Met Gln Gly Ile Val Cys Thr Gly Ile Trp Val Leu Ala His
100 105 110
Glu Cys Gly His Gln Ser Phe Ser Thr Ser Lys Thr Leu Asn Asn Thr
115 120 125
Val Gly Trp Ile Leu His Ser Met Leu Leu Val Pro Tyr His Ser Trp
130 135 140
Arg Ile Ser His Ser Lys His His Lys Ala Thr Gly His Met Thr Lys
145 150 155 160
Asp Gln Val Phe Val Pro Lys Thr Arg Thr Gln Val Gly Leu Pro Pro
165 170 175
Lys Glu Ser Ala Ala Ala Thr Val Gln Glu Glu Glu Asp Met Ser Val
180 185 190
His Leu Asp Glu Glu Ala Pro Ile Val Thr Leu Phe Trp Met Val Ile
195 200 205
Gln Phe Leu Phe Gly Trp Pro Ala Tyr Leu Ile Met Asn Ala Ser Gly
210 215 220
Gln Asp Tyr Gly Arg Trp Thr Ser His Phe His Thr Tyr Ser Pro Ile

225 230 235 240
Phe Glu Pro Arg Asn Phe Phe Asp Ile Ile Leu Ser Asp Leu Gly Val
245 250 255
Leu Ala Thr Leu Gly Ala Leu Ile Tyr Ala Ser Met Gln Leu Ser Leu
260 265 270
Leu Thr Val Thr Lys Tyr Tyr Ile Ile Pro Tyr Leu Phe Val Asn Phe
275 280 285
Trp Leu Val Leu Ile Thr Phe Leu Gln His Thr Asp Pro Lys Leu Pro
290 295 300
His Tyr Arg Glu Gly Ala Trp Asn Phe Gln Arg Gly Ala Leu Cys Thr
305 310 315 320
Val Asp Arg Ser Phe Gly Lys Phe Leu Asp His Met Phe His Gly Ile
325 330 335
Val His Thr His Val Ala His His Leu Phe Ser Gln Met Pro Phe Tyr
340 345 350
His Ala Glu Glu Ala Thr Tyr His Leu Lys Lys Leu Leu Gly Glu Tyr
355 360 365
Tyr Ile Tyr Asp Ala Ser Pro Ile Val Val Ala Val Trp Lys Ser Phe
370 375 380
Arg Glu Cys Arg Phe Val Glu Asp His Gly Asp Val Val Phe Phe Lys
385 390 395 400
Lys

<210> 44
<211> 396
<212> PRT
<213> Mucor rouxii

<400> 44
Met Ala Thr Lys Arg Asn Val Thr Ser Asn Ala Pro Ala Ala Glu Asp
1 5 10 15
Ile Ser Ile Ser Asn Lys Ala Val Ile Asp Glu Ala Ile Glu Arg Asn
20 25 30
Trp Glu Ile Pro Asn Phe Thr Ile Lys Glu Ile Arg Asp Ala Ile Pro
35 40 45
Ala His Cys Phe Arg Arg Asp Thr Phe Arg Ser Phe Thr His Val Leu
50 55 60
His Asp Ile Ile Ile Met Pro Ile Leu Ala Ile Gly Ala Ser Tyr Ile
65 70 75 80
Asp Ser Ile Pro Asn Thr Tyr Ala Arg Ile Ala Leu Trp Pro Leu Tyr
85 90 95
Trp Ile Ala Gln Gly Ile Val Gly Thr Gly Val Trp Val Ile Gly His
100 105 110
Glu Cys Gly His Gln Ala Phe Ser Pro Ser Lys Thr Ile Asn Asn Ser
115 120 125
Val Gly Tyr Val Leu His Thr Ala Leu Leu Val Pro Tyr His Ser Trp
130 135 140
Arg Phe Ser His Ser Lys His His Lys Ala Thr Gly His Met Ser Lys
145 150 155 160
Asp Gln Val Phe Val Pro Ser Thr Arg Lys Glu Tyr Gly Leu Pro Pro
165 170 175
Arg Glu Gln Asp Pro Glu Val Asp Gly Pro His Asp Ala Leu Asp Glu
180 185 190
Val Pro Leu Leu Ser Cys Ile Ala Cys Ser Phe Asn Leu Pro Leu Ala
195 200 205
Gly Leu Phe Ile Ser Ser Pro Met Ser Leu Val Lys Ile Thr Pro Val
210 215 220

Gly Leu Leu Ile Ser Thr Pro Ser Val Leu Ser Thr Ile Glu Asn Gln
225 230 235 240
Phe Trp Asp Val Met Ser Ser Thr Ala Gly Val Leu Gly Met Ile Gly
245 250 255
Phe Leu Ala Tyr Cys Gly Gln Val Leu Ala Leu Leu Leu Ser Ser Ser
260 265 270
Thr Met Leu Phe Pro Tyr Leu Asn Val Asn Phe Trp Leu Val Leu Ile
275 280 285
Thr Tyr Leu Gln His Thr Asp Pro Lys Leu Pro His Tyr Arg Glu Asn
290 295 300
Val Trp Asn Phe Gln Arg Gly Ala Ala Leu Thr Val Asp Arg Ser Tyr
305 310 315 320
Gly Phe Leu Leu Asp Tyr Phe His His His Ile Ser Asp Thr His Val
325 330 335
Ala His His Phe Phe Ser Thr Met Pro His Tyr His Ala Glu Glu Ala
340 345 350
Thr Val His Ile Lys Lys Ala Leu Gly Lys His Tyr His Cys Asp Asn
355 360 365
Thr Pro Val Pro Ile Ala Leu Trp Lys Val Trp Lys Ser Cys Arg Phe
370 375 380
Val Glu Asp Glu Gly Asp Val Val Phe Phe Lys Asn
385 390 395

<210> 45
<211> 396
<212> PRT
<213> Mucor circinelloides

<400> 45
Met Ala Thr Lys Arg Asn Val Thr Ser Asn Ala Pro Ala Ala Glu Asp
1 5 10 15
Ile Ser Ile Ser Asn Lys Ala Val Ile Asp Glu Ala Ile Glu Arg Asn
20 25 30
Trp Glu Ile Pro Asn Phe Thr Ile Lys Glu Ile Arg Asp Ala Ile Pro
35 40 45
Ala His Cys Phe Arg Arg Asp Thr Phe Arg Ser Phe Thr His Val Leu
50 55 60
His Asp Ile Ile Ile Met Ser Ile Leu Ala Ile Gly Ala Ser Tyr Ile
65 70 75 80
Asp Ser Ile Pro Asn Thr Tyr Ala Arg Ile Ala Leu Trp Pro Leu Tyr
85 90 95
Trp Ile Ala Gln Gly Ile Val Gly Thr Gly Val Trp Val Ile Gly His
100 105 110
Glu Cys Gly His Gln Ala Phe Ser Pro Ser Lys Thr Ile Asn Asn Ser
115 120 125
Val Gly Tyr Val Leu His Thr Ala Leu Leu Val Pro Tyr His Ser Trp
130 135 140
Arg Phe Ser His Ser Lys His His Lys Ala Thr Gly His Met Ser Lys
145 150 155 160
Asp Gln Val Phe Val Pro Ser Thr Arg Lys Glu Tyr Gly Leu Pro Pro
165 170 175
Arg Glu Gln Asp Pro Glu Val Asp Gly Pro His Asp Ala Leu Asp Glu
180 185 190
Ala Pro Ile Val Val Leu Tyr Arg Met Phe Leu Gln Phe Thr Phe Gly
195 200 205
Trp Pro Leu Tyr Leu Phe Thr Asn Val Ser Gly Gln Asp Tyr Pro Gly
210 215 220
Trp Ala Ser His Phe Asn Pro Lys Cys Ala Ile Tyr Asp Glu Asn Gln

225 230 235 240
Phe Trp Asp Val Met Ser Ser Thr Ala Gly Val Leu Gly Met Ile Gly
245 250 255
Phe Leu Ala Tyr Cys Gly Gln Val Phe Gly Ser Leu Ala Val Ile Lys
260 265 270
Tyr Tyr Val Ile Pro Tyr Leu Asn Val Asn Phe Trp Leu Val Leu Ile
275 280 285
Thr Tyr Leu Gln His Thr Asp Pro Lys Leu Pro His Tyr Arg Glu Asn
290 295 300
Val Trp Asn Phe Gln Arg Gly Ala Ala Leu Thr Val Asp Arg Ser Tyr
305 310 315 320
Gly Phe Leu Leu Asp Tyr Phe His His His Ile Ser Asp Thr His Val
325 330 335
Ala His His Phe Phe Ser Thr Met Pro His Tyr His Ala Glu Glu Ala
340 345 350
Thr Val His Ile Lys Lys Ala Leu Gly Lys His Tyr His Cys Asp Asn
355 360 365
Thr Pro Val Pro Ile Ala Leu Trp Lys Val Trp Lys Ser Cys Arg Phe
370 375 380
Val Glu Asp Glu Gly Asp Val Val Phe Phe Lys Asn
385 390 395

<210> 46
<211> 424
<212> PRT
<213> Aspergillus fumigatus

<400> 46
Met Ala Ser Asp Ala Glu Lys Thr Ser Ser Lys Met Ile Asp Thr Tyr
1 5 10 15
Gly Asn Glu Phe Lys Ile Pro Asp Tyr Thr Ile Lys Gln Ile Arg Asp
20 25 30
Ala Ile Pro Ala His Cys Tyr Gln Arg Ser Ala Ala Thr Ser Leu Tyr
35 40 45
Tyr Val Phe Arg Asp Met Ala Ile Leu Ala Ser Val Phe Tyr Val Phe
50 55 60
His Asn Tyr Val Thr Pro Glu Thr Val Pro Ser Met Pro Val Arg Val
65 70 75 80
Val Leu Trp Thr Ile Tyr Thr Val Val Gln Gly Leu Val Gly Thr Gly
85 90 95
Val Trp Val Leu Ala His Glu Cys Gly His Gln Ala Phe Ser Thr Ser
100 105 110
Lys Val Leu Asn Asp Thr Val Gly Trp Ile Cys His Ser Leu Leu Leu
115 120 125
Val Pro Tyr Phe Ser Trp Lys Ile Ser His Gly Lys His His Lys Ala
130 135 140
Thr Gly Asn Ile Ala Arg Asp Met Val Phe Val Pro Lys Thr Arg Glu
145 150 155 160
Glu Tyr Ala Thr Arg Ile Gly Arg Ala Ala His Glu Leu Ser Glu Leu
165 170 175
Met Glu Glu Thr Pro Ile Leu Thr Ala Thr Asn Leu Val Leu Gln Gln
180 185 190
Leu Phe Gly Trp Pro Met Tyr Leu Leu Thr Asn Val Thr Gly His Asn
195 200 205
Asn His Glu Arg Gln Pro Glu Gly Arg Gly Lys Gly Lys Arg Asn Gly
210 215 220
Tyr Phe Gly Gly Val Asn His Phe Asn Pro Ser Ser Pro Leu Tyr Glu
225 230 235 240

```
Ala Lys Asp Ala Lys Leu Ile Val Leu Ser Asp Leu Gly Leu Phe Leu
                245                 250                 255
Val Gly Ser Leu Leu Tyr Tyr Ile Gly Ser Thr Tyr Gly Trp Leu Asn
            260                 265                 270
Leu Leu Val Trp Tyr Gly Ile Pro Tyr Leu Trp Val Asn His Trp Leu
        275                 280                 285
Val Ala Ile Thr Phe Leu Gln His Thr Asp Pro Thr Leu Pro His Tyr
    290                 295                 300
Gln Pro Glu Ala Trp Asp Phe Thr Arg Gly Ala Ala Ala Thr Ile Asp
305                 310                 315                 320
Arg Asp Phe Gly Phe Val Gly Arg His Ile Phe His Gly Ile Ile Glu
                325                 330                 335
Thr His Val Leu His His Tyr Val Ser Thr Ile Pro Phe Tyr His Ala
            340                 345                 350
Asp Glu Ala Ser Glu Ala Ile Gln Lys Val Met Gly Pro His Tyr Arg
        355                 360                 365
Ser Glu Ala His Thr Gly Trp Thr Gly Phe Leu Lys Ala Leu Trp Thr
    370                 375                 380
Ser Ala Arg Thr Cys Gln Trp Val Glu Pro Thr Glu Gly Ala Lys Gly
385                 390                 395                 400
Glu Ser Gln Tyr Val Leu Phe Tyr Arg Asn Ile Asn Gly Ile Gly Val
                405                 410                 415
Pro Pro Ala Lys Ile Pro Ala Lys
            420
```

<210> 47
<211> 446
<212> PRT
<213> Cryptococcus curvatus

<400> 47

```
Met Ser Ala Ala Thr Leu Arg Gln Arg Asn Val Asp Lys Pro Gly Ala
 1               5                  10                  15
Ala Asp Lys Ala Glu Leu Leu Arg Glu Ala Glu Asp Leu Glu Leu Thr
            20                  25                  30
Glu Gly Gln Lys Phe Val Gly Pro Asn Phe Thr Val Lys Gln Leu Leu
        35                  40                  45
Asp Ala Ile Pro Ala His Cys Tyr Lys Arg Ser Ala Phe Lys Ser Ser
    50                  55                  60
Leu Tyr Val Leu Gln Asp Phe Val Leu Leu Ala Ala Leu Val Tyr Gly
65                  70                  75                  80
Ala Tyr His Ile Asp Ser Phe Leu Ser Arg Phe Asn Leu Gly Ser Val
                85                  90                  95
Ala His Thr Ala Ala Lys Ile Gly Leu Trp Phe Thr Tyr Gln Val Leu
            100                 105                 110
Ala Gly Met Val Gly Thr Gly Ile Trp Val Ile Ala His Glu Cys Gly
        115                 120                 125
His Gln Ala Tyr Ser Glu Ser Lys Thr Ile Asn Asn Ala Val Gly Trp
    130                 135                 140
Val Leu His Ser Ile Leu Leu Val Pro Tyr His Ser Trp Arg Ile Ser
145                 150                 155                 160
His Gly Arg His His Ala Ala Thr Gly His Leu Thr Arg Asp Glu Val
                165                 170                 175
Phe Val Pro Arg Thr Arg Glu Gln Leu Gly Ile Gln Ala Pro Lys Thr
            180                 185                 190
Glu Glu Glu Lys Lys Gly Ile Asn Val Pro Ala Trp Arg Gln Ala Glu
        195                 200                 205
Leu Arg Glu Ala Leu Glu Glu Ser Pro Ile Gly Ala Leu Tyr Gly Ala
```

```
    210                 215                 220
Ile Leu His Gln Leu Phe Gly Trp Pro Met Tyr Leu Ile Arg Asn Ala
225                 230                 235                 240
Ser Gly Gln Leu Trp Tyr Pro Lys Met Thr Asn His Phe Gln Pro Ser
                245                 250                 255
Ser Ile Ile Phe Lys Pro Ser His Phe Trp Gln Ile Ile Ala Ser Asp
            260                 265                 270
Ile Gly Val Val Leu Thr Ala Ala Ala Leu Gly Val Phe Val Tyr Tyr
        275                 280                 285
Arg Gly Phe Ala Glu Met Ala Arg Ile Tyr Leu Ile Pro Tyr Leu Trp
    290                 295                 300
Val Asn His Trp Leu Val Phe Ile Thr Phe Leu Gln His Thr Asp Pro
305                 310                 315                 320
Val Leu Pro His Tyr Ser Glu Lys Thr Trp Thr Phe Ala Arg Gly Ala
                325                 330                 335
Leu Ala Thr Ile Asp Arg Asn Cys Leu Gly Pro Val Gly Pro Tyr Leu
            340                 345                 350
Phe His Gly Ile Thr Glu Thr His Val Ala His His Thr Ser Ser Arg
        355                 360                 365
Ile Pro His Tyr Asn Ala Trp Glu Ala Thr Glu Ala Leu Lys Lys Phe
    370                 375                 380
Leu Gly Pro His Tyr His Tyr Asn Pro Glu Asn Met Phe Val Ser Phe
385                 390                 395                 400
Trp Lys Ala His Arg Tyr Cys Lys Phe Ile Glu Ala Gly Glu Asp Val
                405                 410                 415
Ala Phe Tyr Arg Asn Ala Ala Gly Val Ala Gln Lys Val Gly Ile Ile
            420                 425                 430
Glu Glu Asn Gly Ala Val Ser Asp Ser Gly Val Glu His Lys
        435                 440                 445
```

<210> 48
<211> 376
<212> PRT
<213> Caenorhabditis elegans

<400> 48

```
Met Thr Ile Ala Thr Lys Val Asn Thr Asn Lys Lys Asp Leu Asp Thr
 1               5                  10                  15
Ile Lys Val Pro Glu Leu Pro Ser Val Ala Ala Val Lys Ala Ala Ile
            20                  25                  30
Pro Glu His Cys Phe Val Lys Asp Pro Leu Thr Ser Ile Ser Tyr Leu
        35                  40                  45
Ile Lys Asp Tyr Val Leu Leu Ala Gly Leu Tyr Phe Ala Val Pro Tyr
    50                  55                  60
Ile Glu His Tyr Leu Gly Trp Ile Gly Leu Leu Gly Trp Tyr Trp Ala
65                  70                  75                  80
Met Gly Ile Val Gly Ser Ala Leu Phe Cys Val Gly His Asp Cys Gly
                85                  90                  95
His Gly Ser Phe Ser Asp Tyr Glu Trp Leu Asn Asp Leu Cys Gly His
            100                 105                 110
Leu Ala His Ala Pro Ile Leu Ala Pro Phe Trp Pro Trp Gln Lys Ser
        115                 120                 125
His Arg Gln His His Gln Tyr Thr Ser His Val Glu Lys Asp Lys Gly
    130                 135                 140
His Pro Trp Val Thr Glu Glu Asp Tyr Asn Asn Arg Thr Ala Ile Glu
145                 150                 155                 160
Lys Tyr Phe Ala Val Ile Pro Ile Ser Gly Trp Leu Arg Trp Asn Pro
                165                 170                 175
```

```
Ile Tyr Thr Ile Val Gly Leu Pro Asp Gly Ser His Phe Trp Pro Trp
            180                 185                 190
Ser Arg Leu Phe Glu Thr Thr Glu Asp Arg Val Lys Cys Ala Val Ser
        195                 200                 205
Gly Val Ala Cys Ala Ile Cys Ala Tyr Ile Ala Phe Val Leu Cys Asp
    210                 215                 220
Tyr Ser Val Tyr Thr Phe Val Lys Tyr Tyr Tyr Ile Pro Leu Leu Phe
225                 230                 235                 240
Gln Gly Leu Ile Leu Val Ile Ile Thr Tyr Leu Gln His Gln Asn Glu
                245                 250                 255
Asp Ile Glu Val Tyr Glu Ala Asp Glu Trp Gly Phe Val Arg Gly Gln
            260                 265                 270
Thr Gln Thr Ile Asp Arg His Trp Gly Phe Gly Leu Asp Asn Ile Met
        275                 280                 285
His Asn Ile Thr Asn Gly His Val Ala His His Phe Phe Phe Thr Lys
    290                 295                 300
Ile Pro His Tyr His Leu Leu Glu Ala Thr Pro Ala Ile Lys Lys Ala
305                 310                 315                 320
Leu Glu Pro Leu Lys Asp Thr Gln Tyr Gly Tyr Lys Arg Glu Val Asn
                325                 330                 335
Tyr Asn Trp Phe Phe Lys Tyr Leu His Tyr Asn Val Thr Leu Asp Tyr
            340                 345                 350
Leu Thr His Lys Ala Lys Gly Val Leu Gln Tyr Arg Ser Gly Val Glu
        355                 360                 365
Ala Ala Lys Ala Lys Lys Ala Gln
    370                 375
```

<210> 49
<211> 457
<212> PRT
<213> Mortierella alpina

<400> 49

```
Met Ala Ala Ala Pro Ser Val Arg Thr Phe Thr Arg Ala Glu Ile Leu
 1               5                  10                  15
Asn Ala Glu Ala Leu Asn Glu Gly Lys Lys Asp Ala Glu Ala Pro Phe
            20                  25                  30
Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe Val Pro
        35                  40                  45
Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys Asp Gly
    50                  55                  60
Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu Thr Leu
65                  70                  75                  80
Ala Asn Phe Tyr Val Gly Asp Ile His Glu Ser Asp Arg Asp Ile Lys
                85                  90                  95
Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu Phe Gln
            100                 105                 110
Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val
        115                 120                 125
Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Phe Val Val Ala Lys
    130                 135                 140
Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Val Ser Ala Ala Leu Leu
145                 150                 155                 160
Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe Leu His
                165                 170                 175
His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe
            180                 185                 190
Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys
```

```
          195                 200                 205
His Asn Thr His His Ala Ala Pro Asn Val His Gly Glu Asp Pro Asp
      210                 215                 220
Ile Asp Thr His Pro Leu Leu Thr Trp Ser Glu His Ala Leu Glu Met
225                 230                 235                 240
Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser Arg Phe
                245                 250                 255
Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala
            260                 265                 270
Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Met Pro Asn Gly
          275                 280                 285
Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu Val Glu
      290                 295                 300
Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr Met Phe
305                 310                 315                 320
Leu Phe Val Lys Asp Pro Ile Asn Met Phe Val Tyr Phe Leu Val Ser
                325                 330                 335
Gln Ala Val Cys Gly Asn Leu Leu Ala Leu Val Phe Ser Leu Asn His
            340                 345                 350
Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met Asp Phe
          355                 360                 365
Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly Leu Phe
      370                 375                 380
Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
385                 390                 395                 400
Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val
                405                 410                 415
Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr Gly Met
            420                 425                 430
Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Arg
          435                 440                 445
Ala Ala Ser Lys Met Gly Lys Ala Gln
      450                 455
```

<210> 50
<211> 523
<212> PRT
<213> Mucor rouxii

<400> 50

```
Met Pro Pro Asn Thr Ala Ala Asp Arg Leu Leu Ser Ser Thr Ser Thr
 1               5                  10                  15
Arg Ser Ser Asn Ile Val Thr Glu Glu Lys Phe Gln Glu Leu Ile Lys
            20                  25                  30
Gln Gly Asp Ser Val Phe Ile Tyr Glu Gln Lys Val Tyr Arg Val Asn
          35                  40                  45
Asn Phe Met Ala Lys His Pro Gly Gly Glu Ala Ala Leu Arg Ser Ala
      50                  55                  60
Leu Gly Arg Asp Val Thr Asp Glu Ile Arg Thr Met His Pro Pro Gln
65                  70                  75                  80
Val Tyr Glu Lys Met Ile Asn Leu Tyr Cys Ile Gly Asp Tyr Met Pro
                85                  90                  95
Asp Val Ile Arg Pro Ala Ser Met Lys Gln Gln His Thr Phe Thr Lys
            100                 105                 110
Pro Lys Glu Asp Lys Pro Val Leu Thr Ala Thr Trp Glu Gly Gly Phe
          115                 120                 125
Thr Val Gln Ala Tyr Asp Asp Ala Ile Gln Asp Leu His Lys His His
      130                 135                 140
```

Ser His Asp Leu Ile Lys Asp Ala Val Leu Gln Lys Asp Leu Asn Gly
145 150 155 160
Asp Gln Ile Arg Asn Ala Tyr Arg Lys Leu Glu Ala Glu Leu Tyr Ala
165 170 175
Lys Gly Leu Phe Lys Cys Asn Tyr Trp Lys Tyr Ala Arg Glu Gly Cys
180 185 190
Arg Tyr Thr Leu Leu Ile Phe Leu Ser Leu Trp Phe Thr Leu Lys Gly
195 200 205
Thr Glu Thr Trp His Tyr Met Ala Gly Ala Ala Phe Met Ala Met Phe
210 215 220
Trp His Gln Leu Val Phe Thr Ala His Asp Ala Gly His Asn Glu Ile
225 230 235 240
Thr Gly Lys Ser Glu Ile Asp His Val Ile Gly Val Ile Ile Ala Asn
245 250 255
Phe Ile Gly Gly Leu Ser Leu Gly Trp Trp Lys Asp Asn His Asn Val
260 265 270
His His Ile Val Thr Asn His Pro Glu His Asp Pro Asp Ile Gln His
275 280 285
Val Pro Phe Met Ala Ile Thr Thr Lys Phe Phe Asn Asn Ile Tyr Ser
290 295 300
Thr Tyr Tyr Lys Arg Val Leu Pro Phe Asp Ala Ala Ser Arg Phe Phe
305 310 315 320
Val Arg His Gln His Tyr Leu Tyr Tyr Leu Ile Leu Ser Phe Gly Arg
325 330 335
Phe Asn Leu His Arg Leu Ser Phe Ala Tyr Leu Leu Thr Cys Lys Asn
340 345 350
Val Arg Thr Arg Thr Leu Glu Leu Val Gly Ile Thr Phe Phe Phe Val
355 360 365
Trp Phe Gly Ser Leu Leu Ser Thr Leu Pro Thr Trp Asn Ile Arg Ile
370 375 380
Ala Tyr Ile Met Val Ser Tyr Met Leu Thr Phe Pro Leu His Val Gln
385 390 395 400
Ile Thr Leu Ser His Phe Gly Met Ser Thr Glu Asp Arg Gly Pro Asp
405 410 415
Glu Pro Phe Pro Ala Lys Met Leu Arg Thr Thr Met Asp Val Asp Cys
420 425 430
Pro Glu Trp Leu Asp Trp Phe His Gly Gly Leu Gln Tyr Gln Ala Val
435 440 445
His His Leu Phe Pro Arg Leu Pro Arg His Asn Leu Arg Gln Cys Val
450 455 460
Pro Leu Val Lys Lys Phe Cys Asp Glu Val Gly Leu His Tyr Tyr Met
465 470 475 480
Tyr Asn Phe Ser Thr Gly Asn Gly Val Val Leu Gly Thr Leu Lys Ser
485 490 495
Val Ala Asp Gln Val Gly Phe Met Asn Glu Val Ala Lys Ser Asn Ala
500 505 510
Glu Ile Trp Ala Asn Asp Lys Glu His Ala His
515 520

<210> 51
<211> 448
<212> PRT
<213> Borago officinalis

<400> 51
Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
1 5 10 15
His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr

```
                20                  25                  30
Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
        35                  40                  45
Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
    50                  55                  60
Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
65                  70                  75                  80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
                85                  90                  95
Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
            100                 105                 110
Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
        115                 120                 125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
    130                 135                 140
Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145                 150                 155                 160
Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
                165                 170                 175
Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
            180                 185                 190
Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
        195                 200                 205
Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
    210                 215                 220
Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
225                 230                 235                 240
Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
                245                 250                 255
Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
            260                 265                 270
Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala His Glu Leu Leu Gly
        275                 280                 285
Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
    290                 295                 300
Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
305                 310                 315                 320
Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
                325                 330                 335
Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
            340                 345                 350
Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
        355                 360                 365
Gly Leu Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
    370                 375                 380
Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
385                 390                 395                 400
His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
                405                 410                 415
Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
            420                 425                 430
Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
        435                 440                 445
```

<210> 52
<211> 446
<212> PRT
<213> Anemone levellei

<400> 52

```
Met Ala Glu Lys Arg Arg Ser Ile Ser Ser Asp Asp Leu Arg Ser His
 1               5                  10                  15
Asn Lys Pro Gly Asp Val Trp Ile Ser Ile Gln Gly Lys Ile Tyr Asp
            20                  25                  30
Val Thr Glu Trp Gly Lys Asp His Pro Gly Gly Glu Gly Pro Leu Leu
        35                  40                  45
Asn Leu Ala Gly Gln Asp Val Thr Asp Ala Phe Val Ala Phe His Pro
    50                  55                  60
Gly Ser Ala Trp Lys Asn Leu Asp Lys Phe His Ile Gly Tyr Leu Gln
65                  70                  75                  80
Asp Tyr Val Val Ser Asp Val Ser Lys Asp Tyr Arg Lys Leu Val Ser
                85                  90                  95
Glu Phe Ser Lys Ala Gly Leu Tyr Glu Lys Lys Gly His Gly His Leu
            100                 105                 110
Ile Arg Leu Leu Val Met Ser Leu Val Phe Ile Ala Ser Val Ser Gly
        115                 120                 125
Val Val Leu Ser Asp Lys Thr Ser Val His Val Gly Ser Ala Val Leu
    130                 135                 140
Leu Ala Val Ile Trp Met Gln Phe Gly Phe Ile Gly His Asp Ser Gly
145                 150                 155                 160
His Tyr Asn Ile Met Thr Ser Pro Glu Leu Asn Arg Tyr Met Gln Ile
                165                 170                 175
Phe Ser Val Asn Val Val Ser Gly Val Ser Val Gly Trp Trp Lys Arg
            180                 185                 190
Tyr His Asn Ala His His Ile Ala Val Asn Ser Leu Glu Tyr Asp Pro
        195                 200                 205
Asp Leu Gln Tyr Val Pro Phe Leu Val Val Ser Thr Ala Ile Phe Asp
    210                 215                 220
Ser Leu Thr Ser His Phe Tyr Arg Lys Lys Met Thr Phe Asp Ala Val
225                 230                 235                 240
Ala Arg Phe Leu Val Ser Phe Gln His Trp Thr Phe Tyr Pro Leu Met
                245                 250                 255
Ala Ile Gly Arg Val Ser Phe Leu Ala Gln Ser Ile Gly Val Leu Leu
            260                 265                 270
Ser Lys Lys Pro Leu Pro Asp Arg His Leu Glu Trp Phe Gly Leu Val
        275                 280                 285
Val Phe Trp Ala Trp Tyr Ser Leu Leu Ile Ser Cys Leu Pro Asn Trp
    290                 295                 300
Trp Glu Arg Val Ile Phe Ile Ala Val Asn Phe Ala Val Thr Gly Ile
305                 310                 315                 320
Gln His Val Gln Phe Cys Leu Asn His Tyr Ser Ala Gln Thr Tyr Ile
                325                 330                 335
Gly Ala Pro Cys Ala Asn Asp Trp Phe Glu Lys Gln Thr Lys Gly Ser
            340                 345                 350
Ile Asp Ile Ser Cys Ser Pro Trp Thr Asp Trp Phe His Gly Gly Leu
        355                 360                 365
Gln Phe Gln Ile Glu His His Leu Phe Pro Arg Met Pro Arg Cys Asn
    370                 375                 380
Leu Arg Lys Ile Ser Pro Phe Val Lys Glu Leu Cys Arg Lys His Asn
385                 390                 395                 400
Leu Val Tyr Thr Ser Val Ser Phe Phe Glu Gly Asn Arg Arg Thr Leu
                405                 410                 415
Ala Thr Leu Lys Asn Ala Ala Leu Lys Ala Arg Asp Leu Thr Ser Pro
            420                 425                 430
Ile Pro Lys Asn Leu Val Trp Glu Ala Val His Thr His Gly
        435                 440                 445
```

<210> 53
<211> 443
<212> PRT
<213> Caenorhabditis elegans

<400> 53

```
Met Val Val Asp Lys Asn Ala Ser Gly Leu Arg Met Lys Val Asp Gly
 1               5                  10                  15
Lys Trp Leu Tyr Leu Ser Glu Glu Leu Val Lys Lys His Pro Gly Gly
            20                  25                  30
Ala Val Ile Glu Gln Tyr Arg Asn Ser Asp Ala Thr His Ile Phe His
        35                  40                  45
Ala Phe His Glu Gly Ser Ser Gln Ala Tyr Lys Gln Leu Asp Leu Leu
    50                  55                  60
Lys Lys His Gly Glu His Asp Glu Phe Leu Glu Lys Gln Leu Glu Lys
65                  70                  75                  80
Arg Leu Asp Lys Val Asp Ile Asn Val Ser Ala Tyr Asp Val Ser Val
                85                  90                  95
Ala Gln Glu Lys Lys Met Val Glu Ser Phe Glu Lys Leu Arg Gln Lys
            100                 105                 110
Leu His Asp Asp Gly Leu Met Lys Ala Asn Glu Thr Tyr Phe Leu Phe
        115                 120                 125
Lys Ala Ile Ser Thr Leu Ser Ile Met Ala Phe Ala Phe Tyr Leu Gln
    130                 135                 140
Tyr Leu Gly Trp Tyr Ile Thr Ser Ala Cys Leu Leu Ala Leu Ala Trp
145                 150                 155                 160
Gln Gln Phe Gly Trp Leu Thr His Glu Phe Cys His Gln Gln Pro Thr
                165                 170                 175
Lys Asn Arg Pro Leu Asn Asp Thr Ile Ser Leu Phe Phe Gly Asn Phe
            180                 185                 190
Leu Gln Gly Phe Ser Arg Asp Trp Trp Lys Asp Lys His Asn Thr His
        195                 200                 205
His Ala Ala Thr Asn Val Ile Asp His Asp Gly Asp Ile Asp Leu Ala
    210                 215                 220
Pro Leu Phe Ala Phe Ile Pro Gly Asp Leu Cys Lys Tyr Lys Ala Ser
225                 230                 235                 240
Phe Glu Lys Ala Ile Leu Lys Ile Val Pro Tyr Gln His Leu Tyr Phe
                245                 250                 255
Thr Ala Met Leu Pro Met Leu Arg Phe Ser Trp Thr Gly Gln Ser Val
            260                 265                 270
Gln Trp Val Phe Lys Glu Asn Gln Met Glu Tyr Lys Val Tyr Gln Arg
        275                 280                 285
Asn Ala Phe Trp Glu Gln Ala Thr Ile Val Gly His Trp Ala Trp Val
    290                 295                 300
Phe Tyr Gln Leu Phe Leu Leu Pro Thr Trp Pro Leu Arg Val Ala Tyr
305                 310                 315                 320
Phe Ile Ile Ser Gln Met Gly Gly Gly Leu Leu Ile Ala His Val Val
                325                 330                 335
Thr Phe Asn His Asn Ser Val Asp Lys Tyr Pro Ala Asn Ser Arg Ile
            340                 345                 350
Leu Asn Asn Phe Ala Ala Leu Gln Ile Leu Thr Thr Arg Asn Met Thr
        355                 360                 365
Pro Ser Pro Phe Ile Asp Trp Leu Trp Gly Gly Leu Asn Tyr Gln Ile
    370                 375                 380
Glu His His Leu Phe Pro Thr Met Pro Arg Cys Asn Leu Asn Ala Cys
385                 390                 395                 400
Met Lys Tyr Val Lys Glu Trp Cys Lys Glu Asn Asn Leu Pro Tyr Leu
                405                 410                 415
```

Val Asp Asp Tyr Phe Asp Gly Tyr Ala Met Asn Leu Gln Gln Leu Lys
420 425 430
Asn Met Ala Glu His Ile Gln Ala Lys Ala Ala
435 440

<210> 54
<211> 318
<212> PRT
<213> Mortierella alpina

<400> 54
Met Glu Ser Ile Ala Gln Phe Leu Pro Ser Lys Met Pro Gln Asp Leu
1 5 10 15
Phe Ile Asp Leu Ala Ala Ala Ile Gly Val Arg Ala Ala Pro Tyr Val
20 25 30
Asp Pro Leu Glu Ala Ala Leu Val Ala Gln Ala Glu Lys Tyr Ile Pro
35 40 45
Thr Ile Val His His Thr Arg Gly Phe Leu Val Ala Val Glu Ser Pro
50 55 60
Leu Val Arg Glu Leu Pro Leu Met Asn Pro Phe His Val Leu Leu Ile
65 70 75 80
Val Leu Ala Tyr Leu Val Thr Val Phe Val Gly Met Gln Ile Met Lys
85 90 95
Asn Phe Asp Arg Phe Glu Val Lys Thr Phe Ser Leu Phe His Asn Phe
100 105 110
Cys Leu Val Ser Ile Ser Ala Tyr Met Cys Gly Gly Ile Leu Tyr Glu
115 120 125
Ala Tyr Gln Ala Asn Tyr Gly Leu Phe Glu Asn Ala Ala Asp His Thr
130 135 140
Ala Lys Gly Phe Pro Met Ala Lys Met Ile Trp Leu Phe Tyr Phe Ser
145 150 155 160
Lys Ile Met Glu Phe Val Asp Thr Met Ile Met Val Leu Lys Lys Asn
165 170 175
Asn Arg Gln Ile Ser Phe Leu His Val Tyr His His Ser Ser Ile Phe
180 185 190
Thr Ile Trp Trp Leu Val Thr Phe Val Ala Pro Asn Gly Glu Ala Tyr
195 200 205
Phe Ser Ala Ala Leu Asn Ser Phe Ile His Val Ile Met Tyr Gly Tyr
210 215 220
Tyr Phe Leu Ser Ala Leu Gly Phe Lys Gln Val Ser Phe Val Lys Phe
225 230 235 240
Tyr Ile Thr Arg Ser Gln Met Thr Gln Phe Cys Met Met Ser Ile Gln
245 250 255
Ser Ser Trp Asp Met Tyr Ala Met Lys Val Leu Gly Arg Pro Gly Tyr
260 265 270
Pro Phe Phe Ile Thr Ala Leu Leu Trp Phe Tyr Met Trp Thr Met Leu
275 280 285
Gly Leu Phe Tyr Asn Phe Tyr Arg Lys Asn Ala Lys Leu Ala Lys Gln
290 295 300
Ala Lys Ala Asp Ala Ala Lys Glu Lys Ala Arg Lys Leu Gln
305 310 315

<210> 55
<211> 290
<212> PRT
<213> Physcomitrella patens

<400> 55
Met Glu Val Val Glu Arg Phe Tyr Gly Glu Leu Asp Gly Lys Val Ser
1 5 10 15
Gln Gly Val Asn Ala Leu Leu Gly Ser Phe Gly Val Glu Leu Thr Asp
20 25 30
Thr Pro Thr Thr Lys Gly Leu Pro Leu Val Asp Ser Pro Thr Pro Ile
35 40 45
Val Leu Gly Val Ser Val Tyr Leu Thr Ile Val Ile Gly Gly Leu Leu
50 55 60
Trp Ile Lys Ala Arg Asp Leu Lys Pro Arg Ala Ser Glu Pro Phe Leu
65 70 75 80
Leu Gln Ala Leu Val Leu Val His Asn Leu Phe Cys Phe Ala Leu Ser
85 90 95
Leu Tyr Met Cys Val Gly Ile Ala Tyr Gln Ala Ile Thr Trp Arg Tyr
100 105 110
Ser Leu Trp Gly Asn Ala Tyr Asn Pro Lys His Lys Glu Met Ala Ile
115 120 125
Leu Val Tyr Leu Phe Tyr Met Ser Lys Tyr Val Glu Phe Met Asp Thr
130 135 140
Val Ile Met Ile Leu Lys Arg Ser Thr Arg Gln Ile Ser Phe Leu His
145 150 155 160
Val Tyr His His Ser Ser Ile Ser Leu Ile Trp Trp Ala Ile Ala His
165 170 175
His Ala Pro Gly Gly Glu Ala Tyr Trp Ser Ala Ala Leu Asn Ser Gly
180 185 190
Val His Val Leu Met Tyr Ala Tyr Tyr Phe Leu Ala Ala Cys Leu Arg
195 200 205
Ser Ser Pro Lys Leu Lys Asn Lys Tyr Leu Phe Trp Gly Arg Tyr Leu
210 215 220
Thr Gln Phe Gln Met Phe Gln Phe Met Leu Asn Leu Val Gln Ala Tyr
225 230 235 240
Tyr Asp Met Lys Thr Asn Ala Pro Tyr Pro Gln Trp Leu Ile Lys Ile
245 250 255
Leu Phe Tyr Tyr Met Ile Ser Leu Leu Phe Leu Phe Gly Asn Phe Tyr
260 265 270
Val Gln Lys Tyr Ile Lys Pro Ser Asp Gly Lys Gln Lys Gly Ala Lys
275 280 285
Thr Glu
290

<210> 56
<211> 288
<212> PRT
<213> Caenorhabditis elegans

<400> 56
Met Ala Gln His Pro Leu Val Gln Arg Leu Leu Asp Val Lys Phe Asp
1 5 10 15
Thr Lys Arg Phe Val Ala Ile Ala Thr His Gly Pro Lys Asn Phe Pro
20 25 30
Asp Ala Glu Gly Arg Lys Phe Phe Ala Asp His Phe Asp Val Thr Ile
35 40 45
Gln Ala Ser Ile Leu Tyr Met Val Val Val Phe Gly Thr Lys Trp Phe
50 55 60
Met Arg Asn Arg Gln Pro Phe Gln Leu Thr Ile Pro Leu Asn Ile Trp
65 70 75 80
Asn Phe Ile Leu Ala Ala Phe Ser Ile Ala Gly Ala Val Lys Met Thr
85 90 95

Pro Glu Phe Phe Gly Thr Ile Ala Asn Lys Gly Ile Val Ala Ser Tyr
100 105 110
Cys Lys Val Phe Asp Phe Thr Lys Gly Glu Asn Gly Tyr Trp Val Trp
115 120 125
Leu Phe Met Ala Ser Lys Leu Phe Glu Leu Val Asp Thr Ile Phe Leu
130 135 140
Val Leu Arg Lys Arg Pro Leu Met Phe Leu His Trp Tyr His His Ile
145 150 155 160
Leu Thr Met Ile Tyr Ala Trp Tyr Ser His Pro Leu Thr Pro Gly Phe
165 170 175
Asn Arg Tyr Gly Ile Tyr Leu Asn Phe Val Val His Ala Phe Met Tyr
180 185 190
Ser Tyr Tyr Phe Leu Arg Ser Met Lys Ile Arg Val Pro Gly Phe Ile
195 200 205
Ala Gln Ala Ile Thr Ser Leu Gln Ile Val Gln Phe Ile Ile Ser Cys
210 215 220
Ala Val Leu Ala His Leu Gly Tyr Leu Met His Phe Thr Asn Ala Asn
225 230 235 240
Cys Asp Phe Glu Pro Ser Val Phe Lys Leu Ala Val Phe Met Asp Thr
245 250 255
Thr Tyr Leu Ala Leu Phe Val Asn Phe Phe Leu Gln Ser Tyr Val Leu
260 265 270
Arg Gly Gly Lys Asp Lys Tyr Lys Ala Val Pro Lys Lys Lys Asn Asn
275 280 285

<210> 57
<211> 299
<212> PRT
<213> Mus musculus

<400> 57
Met Glu His Phe Asp Ala Ser Leu Ser Thr Tyr Phe Lys Ala Phe Leu
1 5 10 15
Gly Pro Arg Asp Thr Arg Val Lys Gly Trp Phe Leu Leu Asp Asn Tyr
20 25 30
Ile Pro Thr Phe Val Cys Ser Val Ile Tyr Leu Leu Ile Val Trp Leu
35 40 45
Gly Pro Lys Tyr Met Lys Asn Arg Gln Pro Phe Ser Cys Arg Gly Ile
50 55 60
Leu Gln Leu Tyr Asn Leu Gly Leu Thr Leu Leu Ser Leu Tyr Met Phe
65 70 75 80
Tyr Glu Leu Val Thr Gly Val Trp Glu Gly Lys Tyr Asn Phe Phe Cys
85 90 95
Gln Gly Thr Arg Ser Ala Gly Glu Ser Asp Met Lys Ile Ile Arg Val
100 105 110
Leu Trp Trp Tyr Tyr Phe Ser Lys Leu Ile Glu Phe Met Asp Thr Phe
115 120 125
Phe Phe Ile Leu Arg Lys Asn Asn His Gln Ile Thr Val Leu His Val
130 135 140
Tyr His His Ala Thr Met Leu Asn Ile Trp Trp Phe Val Met Asn Trp
145 150 155 160
Val Pro Cys Gly His Ser Tyr Phe Gly Ala Thr Leu Asn Ser Phe Ile
165 170 175
His Val Leu Met Tyr Ser Tyr Tyr Gly Leu Ser Ser Ile Pro Ser Met
180 185 190
Arg Pro Tyr Leu Trp Trp Lys Lys Tyr Ile Thr Gln Gly Gln Leu Val
195 200 205
Gln Phe Val Leu Thr Ile Ile Gln Thr Thr Cys Gly Val Phe Trp Pro

```
     210                 215                 220
Cys Ser Phe Pro Leu Gly Trp Leu Phe Phe Gln Ile Gly Tyr Met Ile
225                 230                 235                 240
Ser Leu Ile Ala Leu Phe Thr Asn Phe Tyr Ile Gln Thr Tyr Asn Lys
                245                 250                 255
Lys Gly Ala Ser Arg Arg Lys Asp His Leu Lys Gly His Gln Asn Gly
            260                 265                 270
Ser Val Ala Ala Val Asn Gly His Thr Asn Ser Phe Pro Ser Leu Glu
        275                 280                 285
Asn Ser Val Lys Pro Arg Lys Gln Arg Lys Asp
    290                 295
```

<210> 58
<211> 272
<212> PRT
<213> Thraustochytrium aureum

<400> 58

```
Met Ala Asn Ser Ser Val Trp Asp Asp Val Val Gly Arg Val Glu Thr
 1               5                  10                  15
Gly Val Asp Gln Trp Met Asp Gly Ala Lys Pro Tyr Ala Leu Thr Asp
            20                  25                  30
Gly Leu Pro Met Met Asp Val Ser Thr Met Leu Ala Phe Glu Val Gly
        35                  40                  45
Tyr Met Ala Met Leu Leu Phe Gly Ile Pro Ile Met Lys Gln Met Glu
    50                  55                  60
Lys Pro Phe Glu Leu Lys Thr Ile Lys Leu Leu His Asn Leu Phe Leu
65                  70                  75                  80
Phe Gly Leu Ser Leu Tyr Met Cys Val Glu Thr Ile Arg Gln Ala Ile
                85                  90                  95
Leu Gly Gly Tyr Lys Val Phe Gly Asn Asp Met Glu Lys Gly Asn Glu
            100                 105                 110
Ser His Ala Gln Gly Met Ser Arg Ile Val Tyr Val Phe Tyr Val Ser
        115                 120                 125
Lys Ala Tyr Glu Phe Leu Asp Thr Ala Ile Met Ile Leu Cys Lys Lys
    130                 135                 140
Phe Asn Gln Val Ser Phe Leu His Val Tyr His His Ala Thr Ile Phe
145                 150                 155                 160
Ala Ile Trp Trp Ala Ile Ala Lys Tyr Ala Pro Gly Gly Asp Ala Tyr
                165                 170                 175
Phe Ser Val Ile Leu Asn Ser Phe Val His Thr Val Met Tyr Ala Tyr
            180                 185                 190
Tyr Phe Phe Ser Ser Gln Gly Phe Gly Phe Val Lys Pro Ile Lys Pro
        195                 200                 205
Tyr Ile Thr Thr Leu Gln Met Thr Gln Phe Met Ala Met Leu Val Gln
    210                 215                 220
Ser Leu Tyr Asp Tyr Leu Phe Pro Cys Asp Tyr Pro Gln Ala Leu Val
225                 230                 235                 240
Gln Leu Leu Gly Val Tyr Met Ile Thr Leu Leu Ala Leu Phe Gly Asn
                245                 250                 255
Phe Phe Val Gln Ser Tyr Leu Lys Lys Pro Lys Lys Ser Lys Thr Asn
            260                 265                 270
```

<210> 59
<211> 278
<212> PRT
<213> Phytophthora infestans

<400> 59
Met Ser Thr Glu Leu Leu Gln Ser Tyr Tyr Ala Trp Ala Asn Ala Thr
1 5 10 15
Glu Ala Lys Leu Leu Asp Trp Val Asp Pro Glu Gly Gly Trp Lys Val
20 25 30
His Pro Met Ala Asp Tyr Pro Leu Ala Asn Phe Ser Ser Val Tyr Ala
35 40 45
Ile Cys Val Gly Tyr Leu Leu Phe Val Ile Phe Gly Thr Ala Leu Met
50 55 60
Lys Met Gly Val Pro Ala Ile Lys Thr Ser Pro Leu Gln Phe Val Tyr
65 70 75 80
Asn Pro Ile Gln Val Ile Ala Cys Ser Tyr Met Cys Val Glu Ala Ala
85 90 95
Ile Gln Ala Tyr Arg Asn Gly Tyr Thr Ala Ala Pro Cys Asn Ala Phe
100 105 110
Lys Ser Asp Asp Pro Val Met Gly Asn Val Leu Tyr Leu Phe Tyr Leu
115 120 125
Ser Lys Met Leu Asp Leu Cys Asp Thr Val Phe Ile Ile Leu Gly Lys
130 135 140
Lys Trp Lys Gln Leu Ser Ile Leu His Val Tyr His His Leu Thr Val
145 150 155 160
Leu Phe Val Tyr Tyr Val Thr Phe Arg Ala Ala Gln Asp Gly Asp Ser
165 170 175
Tyr Ala Thr Ile Val Leu Asn Gly Phe Val His Thr Ile Met Tyr Thr
180 185 190
Tyr Tyr Phe Val Ser Ala His Thr Arg Asn Ile Trp Trp Lys Lys Tyr
195 200 205
Leu Thr Arg Ile Gln Leu Ile Gln Phe Val Thr Met Asn Val Gln Gly
210 215 220
Tyr Leu Thr Tyr Ser Arg Gln Cys Pro Gly Met Pro Pro Lys Val Pro
225 230 235 240
Leu Met Tyr Leu Val Tyr Val Gln Ser Leu Phe Trp Leu Phe Met Asn
245 250 255
Phe Tyr Ile Arg Ala Tyr Val Phe Gly Pro Lys Lys Pro Ala Val Glu
260 265 270
Glu Ser Lys Lys Lys Leu
275

<210> 60
<211> 446
<212> PRT
<213> Mortierella alpina

<400> 60
Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Ala Leu Ala Ala
1 5 10 15
His Asn Ala Glu Gly Asp Leu Leu Leu Ala Ile Arg Gly Asn Val Tyr
20 25 30
Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Thr Asp Thr Leu
35 40 45
Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
50 55 60
Glu Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
65 70 75 80
Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His
85 90 95
Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Lys Asp Arg Asn Lys

100 105 110
Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe
115 120 125
Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val
130 135 140
Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe
145 150 155 160
Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe
165 170 175
Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His
180 185 190
Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met
195 200 205
Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val
210 215 220
Ser Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp
225 230 235 240
Phe Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly
245 250 255
Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe
260 265 270
Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His
275 280 285
Thr Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu
290 295 300
Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe
305 310 315 320
Thr Ile Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln
325 330 335
Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn
340 345 350
Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln
355 360 365
Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu
370 375 380
Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His
385 390 395 400
Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asp Thr Cys Ser Glu Tyr Lys
405 410 415
Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His
420 425 430
Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu
435 440 445

<210> 61
<211> 477
<212> PRT
<213> Phytophthora megasperma

<400> 61
Met Ala Pro Ile Glu Thr Val Lys Asp Ala Asn Glu Gly Leu His Gln
1 5 10 15
Arg Lys Gly Ala Ala Ala Ala Ser Lys Asp Thr Thr Thr Phe Thr Trp
20 25 30
Gln Asp Val Ala Lys His Asn Thr Ala Lys Ser Ala Trp Val Thr Ile
35 40 45
Arg Gly Val Val Tyr Asp Val Thr Glu Trp Ala Asp Arg His Pro Gly
50 55 60

Gly Arg Glu Leu Val Leu Leu His Ser Gly Arg Glu Cys Thr Asp Thr
65 70 75 80
Phe Asp Ser Tyr His Pro Phe Ser Asp Arg Ala Asp Lys Ile Leu Ala
85 90 95
Lys Tyr Ala Ile Gly Lys Leu Val Gly Gly Ser Glu Phe Pro Thr Tyr
100 105 110
Lys Pro Asp Thr Gly Phe Tyr Lys Glu Cys Cys Asp Arg Val Asn Gln
115 120 125
Tyr Phe Lys Asp Asn Lys Leu Asp Pro Arg Ser Pro Tyr Ser Gly Leu
130 135 140
Trp Arg Met Ile Leu Val Ala Ile Val Gly Ala Val Ala Tyr Met Gly
145 150 155 160
Met Asn Gln Leu Leu Pro Gly Asn Ile Tyr Ala His Tyr Ala Trp Gly
165 170 175
Ala Leu Phe Gly Val Cys Gln Ala Leu Pro Leu Leu His Val Met His
180 185 190
Asp Ala Ser His Ala Ala Ile Thr Ser Ser Pro Thr Gly Trp Arg Leu
195 200 205
Ile Gly Arg Leu Ala Met Asp Trp Val Ala Gly Ala Asn Met Val Ser
210 215 220
Trp Leu Asn Gln His Val Val Gly His His Ile Tyr Thr Asn Val Ala
225 230 235 240
Gly Ala Asp Pro Asp Leu Pro Val Asp Phe Lys Ser Asp Val Arg Arg
245 250 255
Ile Val Tyr Arg Gln Val Leu Leu Pro Ile Tyr Lys Tyr Gln His Leu
260 265 270
Tyr Leu Pro Pro Leu Tyr Gly Val Leu Gly Leu Lys Phe Arg Val Gln
275 280 285
Asp Val Phe Glu Thr Phe Val Thr Leu Thr Asn Gly Pro Leu Arg Val
290 295 300
Asn Pro Leu Ser Val Gly Asp Trp Ala Glu Met Ile Leu Ser Lys Ala
305 310 315 320
Phe Trp Val Phe Tyr Arg Ile Tyr Leu Pro Leu Ala Val Leu Gln Val
325 330 335
Asp Pro Ala Arg Phe Trp Gly Val Phe Phe Leu Ala Glu Phe Ser Thr
340 345 350
Gly Trp Tyr Leu Ala Phe Asn Phe Gln Val Ser His Val Ser Thr Ala
355 360 365
Cys Glu Tyr Pro Gly Gly Asp Glu Glu Val Thr Ser Ile Asp Asp Glu
370 375 380
Trp Ala Ile Ser Gln Val Lys Ser Ser Val Asp Tyr Gly His Gly Ser
385 390 395 400
Phe Ile Thr Thr Phe Leu Thr Gly Ala Leu Asn Tyr Gln Val Thr His
405 410 415
His Leu Phe Pro Gly Val Ser Gln Tyr His Tyr Pro Ala Ile Ala Pro
420 425 430
Leu Ile Leu Asp Val Cys His Lys Tyr Lys Val Lys Tyr Asn Val Leu
435 440 445
Pro Asp Phe Thr Ala Ala Met Ala Gly His Phe Asp His Leu Val Ile
450 455 460
Met Gly Lys Met Gly Lys Arg Val Thr Ile His Met Gly
465 470 475

<210> 62
<211> 439
<212> PRT
<213> Thraustochytrium sp. ATCC 21685

<400> 62

```
Met Gly Lys Gly Ser Glu Gly Arg Ser Ala Ala Arg Glu Met Thr Ala
 1               5                  10                  15
Glu Ala Asn Gly Asp Lys Arg Lys Thr Ile Leu Ile Glu Gly Val Leu
            20                  25                  30
Tyr Asp Ala Thr Asn Phe Lys His Pro Gly Gly Ser Ile Ile Asn Phe
        35                  40                  45
Leu Thr Glu Gly Glu Ala Gly Val Asp Ala Thr Gln Ala Tyr Arg Glu
    50                  55                  60
Phe His Gln Arg Ser Gly Lys Ala Asp Lys Tyr Leu Lys Ser Leu Pro
65                  70                  75                  80
Lys Leu Asp Ala Ser Lys Val Glu Ser Arg Phe Ser Ala Lys Glu Gln
                85                  90                  95
Ala Arg Arg Asp Ala Met Thr Arg Asp Tyr Ala Ala Phe Arg Glu Glu
            100                 105                 110
Leu Val Ala Glu Gly Tyr Phe Asp Pro Ser Ile Pro His Met Ile Tyr
        115                 120                 125
Arg Val Val Glu Ile Val Ala Leu Phe Ala Leu Ser Phe Trp Leu Met
    130                 135                 140
Ser Lys Ala Ser Pro Thr Ser Leu Val Leu Gly Val Val Met Asn Gly
145                 150                 155                 160
Ile Ala Gln Gly Arg Cys Gly Trp Val Met His Glu Met Gly His Gly
                165                 170                 175
Ser Phe Thr Gly Val Ile Trp Leu Asp Asp Arg Met Cys Glu Phe Phe
            180                 185                 190
Tyr Gly Val Gly Cys Gly Met Ser Gly His Tyr Trp Lys Asn Gln His
        195                 200                 205
Ser Lys His His Ala Ala Pro Asn Arg Leu Glu His Asp Val Asp Leu
    210                 215                 220
Asn Thr Leu Pro Leu Val Ala Phe Asn Glu Arg Val Val Arg Lys Val
225                 230                 235                 240
Lys Pro Gly Ser Leu Leu Ala Leu Trp Leu Arg Val Gln Ala Tyr Leu
                245                 250                 255
Phe Ala Pro Val Ser Cys Leu Leu Ile Gly Leu Gly Trp Thr Leu Tyr
            260                 265                 270
Leu His Pro Arg Tyr Met Leu Arg Thr Lys Arg His Met Glu Phe Val
        275                 280                 285
Trp Ile Phe Ala Arg Tyr Ile Gly Trp Phe Ser Leu Met Gly Ala Leu
    290                 295                 300
Gly Tyr Ser Pro Gly Thr Ser Val Gly Met Tyr Leu Cys Ser Phe Gly
305                 310                 315                 320
Leu Gly Cys Ile Tyr Ile Phe Leu Gln Phe Ala Val Ser His Thr His
                325                 330                 335
Leu Pro Val Thr Asn Pro Glu Asp Gln Leu His Trp Leu Glu Tyr Ala
            340                 345                 350
Ala Asp His Thr Val Asn Ile Ser Thr Lys Ser Trp Leu Val Thr Trp
        355                 360                 365
Trp Met Ser Asn Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Thr
    370                 375                 380
Ala Pro Gln Phe Arg Phe Lys Glu Ile Ser Pro Arg Val Glu Ala Leu
385                 390                 395                 400
Phe Lys Arg His Asn Leu Pro Tyr Tyr Asp Leu Pro Tyr Thr Ser Ala
                405                 410                 415
Val Ser Thr Thr Phe Ala Asn Leu Tyr Ser Val Gly His Ser Val Gly
            420                 425                 430
Ala Asp Thr Lys Lys Gln Asp
        435
```

<210> 63
<211> 447
<212> PRT
<213> Caenorhabditis elegans

<400> 63

```
Met Val Leu Arg Glu Gln Glu His Glu Pro Phe Phe Ile Lys Ile Asp
 1               5                  10                  15
Gly Lys Trp Cys Gln Ile Asp Asp Ala Val Leu Arg Ser His Pro Gly
            20                  25                  30
Gly Ser Ala Ile Thr Thr Tyr Lys Asn Met Asp Ala Thr Thr Val Phe
        35                  40                  45
His Thr Phe His Thr Gly Ser Lys Glu Ala Tyr Gln Trp Leu Thr Glu
    50                  55                  60
Leu Lys Lys Glu Cys Pro Thr Gln Glu Pro Glu Ile Pro Asp Ile Lys
65                  70                  75                  80
Asp Asp Pro Ile Lys Gly Ile Asp Asp Val Asn Met Gly Thr Phe Asn
                85                  90                  95
Ile Ser Glu Lys Arg Ser Ala Gln Ile Asn Lys Ser Phe Thr Asp Leu
            100                 105                 110
Arg Met Arg Val Arg Ala Glu Gly Leu Met Asp Gly Ser Pro Leu Phe
        115                 120                 125
Tyr Ile Arg Lys Ile Leu Glu Thr Ile Phe Thr Ile Leu Phe Ala Phe
    130                 135                 140
Tyr Leu Gln Tyr His Thr Tyr Tyr Leu Pro Ser Ala Ile Leu Met Gly
145                 150                 155                 160
Val Ala Trp Gln Gln Leu Gly Trp Leu Ile His Glu Phe Ala His His
                165                 170                 175
Gln Leu Phe Lys Asn Arg Tyr Tyr Asn Asp Leu Ala Ser Tyr Phe Val
            180                 185                 190
Gly Asn Phe Leu Gln Gly Phe Ser Ser Gly Gly Trp Lys Glu Gln His
        195                 200                 205
Asn Val His His Ala Ala Thr Asn Val Val Gly Arg Asp Gly Asp Leu
    210                 215                 220
Asp Leu Val Pro Phe Tyr Ala Thr Val Ala Glu His Leu Asn Asn Tyr
225                 230                 235                 240
Ser Gln Asp Ser Trp Val Met Thr Leu Phe Arg Trp Gln His Val His
                245                 250                 255
Trp Thr Phe Met Leu Pro Phe Leu Arg Leu Ser Trp Leu Leu Gln Ser
            260                 265                 270
Ile Ile Phe Val Ser Gln Met Pro Thr His Tyr Tyr Asp Tyr Tyr Arg
        275                 280                 285
Asn Thr Ala Ile Tyr Glu Gln Val Gly Leu Ser Leu His Trp Ala Trp
    290                 295                 300
Ser Leu Gly Gln Leu Tyr Phe Leu Pro Asp Trp Ser Thr Arg Ile Met
305                 310                 315                 320
Phe Phe Leu Val Ser His Leu Val Gly Gly Phe Leu Leu Ser His Val
                325                 330                 335
Val Thr Phe Asn His Tyr Ser Val Glu Lys Phe Ala Leu Ser Ser Asn
            340                 345                 350
Ile Met Ser Asn Tyr Ala Cys Leu Gln Ile Met Thr Thr Arg Asn Met
        355                 360                 365
Arg Pro Gly Arg Phe Ile Asp Trp Leu Trp Gly Gly Leu Asn Tyr Gln
    370                 375                 380
Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Leu Asn Thr
385                 390                 395                 400
Val Met Pro Leu Val Lys Glu Phe Ala Ala Ala Asn Gly Leu Pro Tyr
                405                 410                 415
Met Val Asp Asp Tyr Phe Thr Gly Phe Trp Leu Glu Ile Glu Gln Phe
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                420                 425                 430
Arg Asn Ile Ala Asn Val Ala Ala Lys Leu Thr Lys Lys Ile Ala
        435                 440                 445
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<210> 64
<211> 456
<212> PRT
<213> Pythium irregulare

<400> 64

```
Met Thr Glu Lys Ala Ser Asp Glu Phe Thr Trp Gln Glu Val Ala Lys
 1               5                  10                  15
His Asn Thr Ala Lys Ser Ala Trp Val Ile Ile Arg Gly Glu Val Tyr
            20                  25                  30
Asp Val Thr Glu Trp Ala Asp Lys His Pro Gly Gly Ser Glu Leu Ile
        35                  40                  45
Val Leu His Ser Gly Arg Glu Cys Thr Asp Thr Phe Tyr Ser Tyr His
    50                  55                  60
Pro Phe Ser Asn Arg Ala Asp Lys Ile Leu Ala Lys Tyr Lys Ile Gly
65                  70                  75                  80
Lys Leu Val Gly Gly Tyr Glu Phe Pro Val Phe Lys Pro Asp Ser Gly
                85                  90                  95
Phe Tyr Lys Glu Cys Ser Glu Arg Val Ala Glu Tyr Phe Lys Thr Asn
            100                 105                 110
Asn Leu Asp Pro Lys Ala Ala Phe Ala Gly Leu Trp Arg Met Val Phe
        115                 120                 125
Val Phe Ala Val Ala Ala Leu Ala Tyr Met Gly Met Asn Glu Leu Ile
    130                 135                 140
Pro Gly Asn Val Tyr Ala Gln Tyr Ala Trp Gly Val Val Phe Gly Val
145                 150                 155                 160
Phe Gln Ala Leu Pro Leu Leu His Val Met His Asp Ser Ser His Ala
                165                 170                 175
Ala Cys Ser Ser Ser Pro Ala Met Trp Gln Ile Ile Gly Arg Gly Val
            180                 185                 190
Met Asp Trp Phe Ala Gly Ala Ser Met Val Ser Trp Leu Asn Gln His
        195                 200                 205
Val Val Gly His His Ile Tyr Thr Asn Val Ala Gly Ala Asp Pro Asp
    210                 215                 220
Leu Pro Val Asp Phe Glu Ser Asp Val Arg Arg Ile Val His Arg Gln
225                 230                 235                 240
Val Leu Leu Pro Ile Tyr Lys Phe Gln His Ile Tyr Leu Pro Pro Leu
                245                 250                 255
Tyr Gly Val Leu Gly Leu Lys Phe Arg Ile Gln Asp Val Phe Glu Thr
            260                 265                 270
Phe Val Ser Leu Thr Asn Gly Pro Val Arg Val Asn Pro His Pro Val
        275                 280                 285
Ser Asp Trp Val Gln Met Ile Phe Ala Lys Ala Phe Trp Thr Phe Tyr
    290                 295                 300
Arg Ile Tyr Ile Pro Leu Ala Trp Leu Lys Ile Thr Pro Ser Thr Phe
305                 310                 315                 320
Trp Gly Val Phe Phe Leu Ala Glu Phe Thr Thr Gly Trp Tyr Leu Ala
                325                 330                 335
Phe Asn Phe Gln Val Ser His Val Ser Thr Glu Cys Glu Tyr Pro Cys
            340                 345                 350
Gly Asp Ala Pro Ser Ala Glu Val Gly Asp Glu Trp Ala Ile Ser Gln
        355                 360                 365
Val Lys Ser Ser Val Asp Tyr Ala His Gly Ser Pro Leu Ala Ala Phe
    370                 375                 380
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Leu Cys Gly Ala Leu Asn Tyr Gln Val Thr His His Leu Tyr Pro Gly
385 390 395 400
Ile Ser Gln Tyr His Tyr Pro Ala Ile Ala Pro Ile Ile Ile Asp Val
405 410 415
Cys Lys Lys Tyr Asn Ile Lys Tyr Thr Val Leu Pro Thr Phe Thr Glu
420 425 430
Ala Leu Leu Ala His Phe Lys His Leu Lys Asn Met Gly Glu Leu Gly
435 440 445
Lys Pro Val Glu Ile His Met Gly
450 455

<210> 65
<211> 469
<212> PRT
<213> Phaeodactylum tricornutum

<400> 65
Met Ala Pro Asp Ala Asp Lys Leu Arg Gln Arg Gln Thr Thr Ala Val
1 5 10 15
Ala Lys His Asn Ala Ala Thr Ile Ser Thr Gln Glu Arg Leu Cys Ser
20 25 30
Leu Ser Ser Leu Lys Gly Glu Glu Val Cys Ile Asp Gly Ile Ile Tyr
35 40 45
Asp Leu Gln Ser Phe Asp His Pro Gly Gly Glu Thr Ile Lys Met Phe
50 55 60
Gly Gly Asn Asp Val Thr Val Gln Tyr Lys Met Ile His Pro Tyr His
65 70 75 80
Thr Glu Lys His Leu Glu Lys Met Lys Arg Val Gly Lys Val Thr Asp
85 90 95
Phe Val Cys Glu Tyr Lys Phe Asp Thr Glu Phe Glu Arg Glu Ile Lys
100 105 110
Arg Glu Val Phe Lys Ile Val Arg Arg Gly Lys Asp Phe Gly Thr Leu
115 120 125
Gly Trp Phe Phe Arg Ala Phe Cys Tyr Ile Ala Ile Phe Phe Tyr Leu
130 135 140
Gln Tyr His Trp Val Thr Thr Gly Thr Ser Trp Leu Leu Ala Val Ala
145 150 155 160
Tyr Gly Ile Ser Gln Ala Met Ile Gly Met Asn Val Gln His Asp Ala
165 170 175
Asn His Gly Ala Thr Ser Lys Arg Pro Trp Val Asn Asp Met Leu Gly
180 185 190
Leu Gly Ala Asp Phe Ile Gly Gly Ser Lys Trp Leu Trp Gln Glu Gln
195 200 205
His Trp Thr His His Ala Tyr Thr Asn His Ala Glu Met Asp Pro Asp
210 215 220
Ser Phe Gly Ala Glu Pro Met Leu Leu Phe Asn Asp Tyr Pro Leu Asp
225 230 235 240
His Pro Ala Arg Thr Trp Leu His Arg Phe Gln Ala Phe Phe Tyr Met
245 250 255
Pro Val Leu Ala Gly Tyr Trp Leu Ser Ala Val Phe Asn Pro Gln Ile
260 265 270
Leu Asp Leu Gln Gln Arg Gly Ala Leu Ser Val Gly Ile Arg Leu Asp
275 280 285
Asn Ala Phe Ile His Ser Arg Arg Lys Tyr Ala Val Phe Trp Arg Ala
290 295 300
Val Tyr Ile Ala Val Asn Val Ile Ala Pro Phe Tyr Thr Asn Ser Gly
305 310 315 320
Leu Glu Trp Ser Trp Arg Val Phe Gly Asn Ile Met Leu Met Gly Val

325 330 335
Ala Glu Ser Leu Ala Leu Ala Val Leu Phe Ser Leu Ser His Asn Phe
340 345 350
Glu Ser Ala Asp Arg Asp Pro Thr Ala Pro Leu Lys Lys Thr Gly Glu
355 360 365
Pro Val Asp Trp Phe Lys Thr Gln Val Glu Thr Ser Cys Thr Tyr Gly
370 375 380
Gly Phe Leu Ser Gly Cys Phe Thr Gly Gly Leu Asn Phe Gln Val Glu
385 390 395 400
His His Leu Phe Pro Arg Met Ser Ser Ala Trp Tyr Pro Tyr Ile Ala
405 410 415
Pro Lys Val Arg Glu Ile Cys Ala Lys His Gly Val His Tyr Ala Tyr
420 425 430
Tyr Pro Trp Ile His Gln Asn Phe Leu Ser Thr Val Arg Tyr Met His
435 440 445
Ala Ala Gly Thr Gly Ala Asn Trp Arg Gln Met Ala Arg Glu Asn Pro
450 455 460
Leu Thr Gly Arg Ala
465

<210> 66
<211> 292
<212> PRT
<213> Mus musculus

<400> 66
Met Glu Gln Leu Lys Ala Phe Asp Asn Glu Val Asn Ala Phe Leu Asp
1 5 10 15
Asn Met Phe Gly Pro Arg Asp Ser Arg Val Arg Gly Trp Phe Leu Leu
20 25 30
Asp Ser Tyr Leu Pro Thr Phe Ile Leu Thr Ile Thr Tyr Leu Leu Ser
35 40 45
Ile Trp Leu Gly Asn Lys Tyr Met Lys Asn Arg Pro Ala Leu Ser Leu
50 55 60
Arg Gly Ile Leu Thr Leu Tyr Asn Leu Ala Ile Thr Leu Leu Ser Ala
65 70 75 80
Tyr Met Leu Val Glu Leu Ile Leu Ser Ser Trp Glu Gly Gly Tyr Asn
85 90 95
Leu Gln Cys Gln Asn Leu Asp Ser Ala Gly Glu Gly Asp Val Arg Val
100 105 110
Ala Lys Val Leu Trp Trp Tyr Tyr Phe Ser Lys Leu Val Glu Phe Leu
115 120 125
Asp Thr Ile Phe Phe Val Leu Arg Lys Lys Thr Asn Gln Ile Thr Phe
130 135 140
Leu His Val Tyr His His Ala Ser Met Phe Asn Ile Trp Trp Cys Val
145 150 155 160
Leu Asn Trp Ile Pro Cys Gly Gln Ser Phe Phe Gly Pro Thr Leu Asn
165 170 175
Ser Phe Ile His Ile Leu Met Tyr Ser Tyr Tyr Gly Leu Ser Val Phe
180 185 190
Pro Ser Met His Lys Tyr Leu Trp Trp Lys Lys Tyr Leu Thr Gln Ala
195 200 205
Gln Leu Val Gln Phe Val Leu Thr Ile Thr His Thr Leu Ser Ala Val
210 215 220
Val Lys Pro Cys Gly Phe Pro Phe Gly Cys Leu Ile Phe Gln Ser Ser
225 230 235 240
Tyr Met Met Thr Leu Val Ile Leu Phe Leu Asn Phe Tyr Ile Gln Thr
245 250 255

Tyr Arg Lys Lys Pro Val Lys Lys Glu Leu Gln Glu Lys Glu Val Lys
260 265 270
Asn Gly Phe Pro Lys Ala His Leu Ile Val Ala Asn Gly Met Thr Asp
275 280 285
Lys Lys Ala Gln
290

<210> 67
<211> 299
<212> PRT
<213> Homo sapiens

<400> 67
Met Glu His Phe Asp Ala Ser Leu Ser Thr Tyr Phe Lys Ala Leu Leu
1 5 10 15
Gly Pro Arg Asp Thr Arg Val Lys Gly Trp Phe Leu Leu Asp Asn Tyr
20 25 30
Ile Pro Thr Phe Ile Cys Ser Val Ile Tyr Leu Leu Ile Val Trp Leu
35 40 45
Gly Pro Lys Tyr Met Arg Asn Lys Gln Pro Phe Ser Cys Arg Gly Ile
50 55 60
Leu Val Val Tyr Asn Leu Gly Leu Thr Leu Leu Ser Leu Tyr Met Phe
65 70 75 80
Cys Glu Leu Val Thr Gly Val Trp Glu Gly Lys Tyr Asn Phe Phe Cys
85 90 95
Gln Gly Thr Arg Thr Ala Gly Glu Ser Asp Met Lys Ile Ile Arg Val
100 105 110
Leu Trp Trp Tyr Tyr Phe Ser Lys Leu Ile Glu Phe Met Asp Thr Phe
115 120 125
Phe Phe Ile Leu Arg Lys Asn Asn His Gln Ile Thr Val Leu His Val
130 135 140
Tyr His His Ala Ser Met Leu Asn Ile Trp Trp Phe Val Met Asn Trp
145 150 155 160
Val Pro Cys Gly His Ser Tyr Phe Gly Ala Thr Leu Asn Ser Phe Ile
165 170 175
His Val Leu Met Tyr Ser Tyr Tyr Gly Leu Ser Ser Val Pro Ser Met
180 185 190
Arg Pro Tyr Leu Trp Trp Lys Lys Tyr Ile Thr Gln Gly Gln Leu Leu
195 200 205
Gln Phe Val Leu Thr Ile Ile Gln Thr Ser Cys Gly Val Ile Trp Pro
210 215 220
Cys Thr Phe Pro Leu Gly Trp Leu Tyr Phe Gln Ile Gly Tyr Met Ile
225 230 235 240
Ser Leu Ile Ala Leu Phe Thr Asn Phe Tyr Ile Gln Thr Tyr Asn Lys
245 250 255
Lys Gly Ala Ser Arg Arg Lys Asp His Leu Lys Asp His Gln Asn Gly
260 265 270
Ser Met Ala Ala Val Asn Gly His Thr Asn Ser Phe Ser Pro Leu Glu
275 280 285
Asn Asn Val Lys Pro Arg Lys Leu Arg Lys Asp
290 295

<210> 68
<211> 277
<212> PRT
<213> Pavlova

<400> 68
Met Met Leu Ala Ala Gly Tyr Leu Leu Val Leu Ser Ala Ala Arg Gln
1 5 10 15
Ser Phe Gln Gln Asp Ile Asp Asn Pro Asn Gly Ala Tyr Ser Thr Ser
20 25 30
Trp Thr Gly Leu Pro Ile Val Met Ser Val Val Tyr Leu Ser Gly Val
35 40 45
Phe Gly Leu Thr Lys Tyr Phe Glu Asn Arg Lys Pro Met Thr Gly Leu
50 55 60
Lys Asp Tyr Met Phe Thr Tyr Asn Leu Tyr Gln Val Ile Ile Asn Val
65 70 75 80
Trp Cys Val Val Ala Phe Leu Leu Glu Val Arg Arg Ala Gly Met Ser
85 90 95
Leu Ile Gly Asn Lys Val Asp Leu Gly Pro Asn Ser Phe Arg Leu Gly
100 105 110
Phe Val Thr Trp Val His Tyr Asn Asn Lys Tyr Val Glu Leu Leu Asp
115 120 125
Thr Leu Trp Met Val Leu Arg Lys Lys Thr Gln Gln Val Ser Phe Leu
130 135 140
His Val Tyr His His Val Leu Leu Met Trp Ala Trp Phe Val Val Val
145 150 155 160
Lys Leu Gly Asn Gly Gly Asp Ala Tyr Phe Gly Gly Leu Met Asn Ser
165 170 175
Ile Ile His Val Met Met Tyr Ser Tyr Tyr Thr Met Ala Leu Leu Gly
180 185 190
Trp Ser Cys Pro Trp Lys Arg Tyr Leu Thr Gln Ala Gln Leu Val Gln
195 200 205
Phe Cys Ile Cys Leu Ala His Ser Thr Trp Ala Ala Val Thr Gly Ala
210 215 220
Tyr Pro Trp Arg Ile Cys Leu Val Glu Val Trp Val Met Val Ser Met
225 230 235 240
Leu Val Leu Phe Thr Arg Phe Tyr Arg Gln Ala Tyr Ala Lys Glu Ala
245 250 255
Lys Ala Lys Glu Ala Lys Lys Leu Ala Gln Glu Ala Ser Gln Ala Lys
260 265 270
Ala Val Lys Ala Glu
275

<210> 69
<211> 402
<212> PRT
<213> Caenorhabditis elegans

<400> 69
Met Val Ala His Ser Ser Glu Gly Leu Ser Ala Thr Ala Pro Val Thr
1 5 10 15
Gly Gly Asp Val Leu Val Asp Ala Arg Ala Ser Leu Glu Glu Lys Glu
20 25 30
Ala Pro Arg Asp Val Asn Ala Asn Thr Lys Gln Ala Thr Thr Glu Glu
35 40 45
Pro Arg Ile Gln Leu Pro Thr Val Asp Ala Phe Arg Arg Ala Ile Pro
50 55 60
Ala His Cys Phe Glu Arg Asp Leu Val Lys Ser Ile Arg Tyr Leu Val
65 70 75 80
Gln Asp Phe Ala Ala Leu Thr Ile Leu Tyr Phe Ala Leu Pro Ala Phe
85 90 95
Glu Tyr Phe Gly Leu Phe Gly Tyr Leu Val Trp Asn Ile Phe Met Gly
100 105 110

Val Phe Gly Phe Ala Leu Phe Val Val Gly His Asp Cys Leu His Gly
115 120 125
Ser Phe Ser Asp Asn Gln Asn Leu Asn Asp Phe Ile Gly His Ile Ala
130 135 140
Phe Ser Pro Leu Phe Ser Pro Tyr Phe Pro Trp Gln Lys Ser His Lys
145 150 155 160
Leu His His Ala Phe Thr Asn His Ile Asp Lys Asp His Gly His Val
165 170 175
Trp Ile Gln Asp Lys Asp Trp Glu Ala Met Pro Ser Trp Lys Arg Trp
180 185 190
Phe Asn Pro Ile Pro Phe Ser Gly Trp Leu Lys Trp Phe Pro Val Tyr
195 200 205
Thr Leu Phe Gly Phe Cys Asp Gly Ser His Phe Trp Pro Tyr Ser Ser
210 215 220
Leu Phe Val Arg Asn Ser Glu Arg Val Gln Cys Val Ile Ser Gly Ile
225 230 235 240
Cys Cys Cys Val Cys Ala Tyr Ile Ala Leu Thr Ile Ala Gly Ser Tyr
245 250 255
Ser Asn Trp Phe Trp Tyr Tyr Trp Val Pro Leu Ser Phe Phe Gly Leu
260 265 270
Met Leu Val Ile Val Thr Tyr Leu Gln His Val Asp Asp Val Ala Glu
275 280 285
Val Tyr Glu Ala Asp Glu Trp Ser Phe Val Arg Gly Gln Thr Gln Thr
290 295 300
Ile Asp Arg Tyr Tyr Gly Leu Gly Leu Asp Thr Thr Met His His Ile
305 310 315 320
Thr Asp Gly His Val Ala His His Phe Phe Asn Lys Ile Pro His Tyr
325 330 335
His Leu Ile Glu Ala Thr Glu Gly Val Lys Lys Val Leu Glu Pro Leu
340 345 350
Ser Asp Thr Gln Tyr Gly Tyr Lys Ser Gln Val Asn Tyr Asp Phe Phe
355 360 365
Ala Arg Phe Leu Trp Phe Asn Tyr Lys Leu Asp Tyr Leu Val His Lys
370 375 380
Thr Ala Gly Ile Met Gln Phe Arg Thr Thr Leu Glu Glu Lys Ala Lys
385 390 395 400
Ala Lys

<210> 70
<211> 438
<212> PRT
<213> Petroselinum crispum

<400> 70
Met Ala Ser Trp Val Ile Ser Glu Cys Gly Leu Arg Pro Leu Pro Arg
1 5 10 15
Ile Tyr Ala Arg Pro Arg Ser Gly Ala Ser Cys Phe Asn Ser Lys Asn
20 25 30
Pro Val Lys Asn Leu Arg Phe Leu Asp Glu Asn Val Lys Ile Ser Met
35 40 45
Thr Gly Ser Arg Asn Trp Gly Leu Arg Val Ser Val Pro Met Ser Val
50 55 60
Pro Ser Val Ser Glu Glu Glu Glu Arg Phe Glu Ser Leu Val Glu Glu
65 70 75 80
Glu Asn Glu Phe Asp Pro Gly Ala Ala Pro Pro Phe Lys Leu Ser Asp
85 90 95
Val Arg Ala Ala Ile Pro Lys His Cys Trp Val Lys Asp Pro Val Arg

100 105 110
Ser Met Ser Tyr Val Leu Arg Asp Val Leu Ile Val Phe Gly Leu Ala
115 120 125
Val Ala Ala Ser Phe Val Asn Asn Trp Ala Val Trp Pro Leu Tyr Trp
130 135 140
Ile Ala Gln Gly Thr Met Phe Trp Ala Leu Phe Val Leu Gly His Asp
145 150 155 160
Cys Gly His Gly Ser Phe Ser Asn Asp Ala Lys Leu Asn Ser Val Val
165 170 175
Gly His Ile Leu His Ser Ser Ile Leu Val Pro Tyr His Gly Trp Arg
180 185 190
Ile Ser His Arg Thr His His Gln Asn His Gly His Val Glu Asn Asp
195 200 205
Glu Ser Trp His Pro Leu Ser Glu Lys Leu Phe Asn Ser Leu Asp Asp
210 215 220
Leu Thr Arg Lys Phe Arg Phe Thr Leu Pro Phe Pro Met Leu Ala Tyr
225 230 235 240
Pro Phe Tyr Leu Trp Gly Arg Ser Pro Gly Lys Lys Gly Ser His Tyr
245 250 255
Asp Pro Ser Ser Asp Leu Phe Val Pro Asn Glu Arg Lys Asp Val Ile
260 265 270
Thr Ser Thr Val Cys Trp Thr Ala Met Ala Ala Leu Leu Val Gly Leu
275 280 285
Asn Phe Val Met Gly Pro Val Lys Met Leu Met Leu Tyr Gly Ile Pro
290 295 300
Tyr Trp Ile Phe Val Met Trp Leu Asp Phe Val Thr Tyr Leu His His
305 310 315 320
His Gly His Asp Asp Lys Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser
325 330 335
Tyr Leu Arg Gly Gly Leu Thr Thr Leu Asp Arg Asp Tyr Gly Trp Ile
340 345 350
Asn Asn Ile His His Asp Ile Gly Thr His Val Val His His Leu Phe
355 360 365
Pro Gln Ile Pro His Tyr His Leu Ile Glu Ala Thr Glu Ala Ala Lys
370 375 380
Pro Val Phe Gly Lys Tyr Tyr Arg Glu Pro Lys Lys Ser Gly Pro Val
385 390 395 400
Pro Phe His Leu Leu Ala Thr Leu Trp Lys Ser Phe Lys Lys Asp His
405 410 415
Phe Val Ser Asp Thr Gly Asp Val Val Tyr Tyr Gln Ala His Pro Glu
420 425 430
Ile Ala Lys Thr Gln Lys
435

<210> 71
<211> 446
<212> PRT
<213> Arabidopsis thaliana

<400> 71
Met Ala Asn Leu Val Leu Ser Glu Cys Gly Ile Arg Pro Leu Pro Arg
1 5 10 15
Ile Tyr Thr Thr Pro Arg Ser Asn Phe Leu Ser Asn Asn Asn Lys Phe
20 25 30
Arg Pro Ser Leu Ser Ser Ser Ser Tyr Lys Thr Ser Ser Ser Pro Leu
35 40 45
Ser Phe Gly Leu Asn Ser Arg Asp Gly Phe Thr Arg Asn Trp Ala Leu
50 55 60

```
Asn Val Ser Thr Pro Leu Thr Thr Pro Ile Phe Glu Glu Ser Pro Leu
65                  70                  75                  80
Glu Glu Asp Asn Lys Gln Arg Phe Asp Pro Gly Ala Pro Pro Pro Phe
                85                  90                  95
Asn Leu Ala Asp Ile Arg Ala Ala Ile Pro Lys His Cys Trp Val Lys
            100                 105                 110
Asn Pro Trp Lys Ser Leu Ser Tyr Val Val Arg Asp Val Ala Ile Val
        115                 120                 125
Phe Ala Leu Ala Ala Gly Ala Ala Tyr Leu Asn Asn Trp Ile Val Trp
    130                 135                 140
Pro Leu Tyr Trp Leu Ala Gln Gly Thr Met Phe Trp Ala Leu Phe Val
145                 150                 155                 160
Leu Gly His Asp Cys Gly His Gly Ser Phe Ser Asn Asp Pro Lys Leu
                165                 170                 175
Asn Ser Val Val Gly His Leu Leu His Ser Ser Ile Leu Val Pro Tyr
            180                 185                 190
His Gly Trp Arg Ile Ser His Arg Thr His His Gln Asn His Gly His
        195                 200                 205
Val Glu Asn Asp Glu Ser Trp His Pro Met Ser Glu Lys Ile Tyr Asn
    210                 215                 220
Thr Leu Asp Lys Pro Thr Arg Phe Phe Arg Phe Thr Leu Pro Leu Val
225                 230                 235                 240
Met Leu Ala Tyr Pro Phe Tyr Leu Trp Ala Arg Ser Pro Gly Lys Lys
                245                 250                 255
Gly Ser His Tyr His Pro Asp Ser Asp Leu Phe Leu Pro Lys Glu Arg
            260                 265                 270
Lys Asp Val Leu Thr Ser Thr Ala Cys Trp Thr Ala Met Ala Ala Leu
        275                 280                 285
Leu Val Cys Leu Asn Phe Thr Ile Gly Pro Ile Gln Met Leu Lys Leu
    290                 295                 300
Tyr Gly Ile Pro Tyr Trp Ile Asn Val Met Trp Leu Asp Phe Val Thr
305                 310                 315                 320
Tyr Leu His His His Gly His Glu Asp Lys Leu Pro Trp Tyr Arg Gly
                325                 330                 335
Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu Thr Thr Leu Asp Arg Asp
            340                 345                 350
Tyr Gly Leu Ile Asn Asn Ile His His Asp Ile Gly Thr His Val Ile
        355                 360                 365
His His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Glu Ala Thr
    370                 375                 380
Glu Ala Ala Lys Pro Val Leu Gly Lys Tyr Tyr Arg Glu Pro Asp Lys
385                 390                 395                 400
Ser Gly Pro Leu Pro Leu His Leu Leu Glu Ile Leu Ala Lys Ser Ile
                405                 410                 415
Lys Glu Asp His Tyr Val Ser Asp Glu Gly Glu Val Val Tyr Tyr Lys
            420                 425                 430
Ala Asp Pro Asn Leu Tyr Gly Glu Val Lys Val Arg Ala Asp
        435                 440                 445
```

<210> 72
<211> 377
<212> PRT
<213> Brassica napus

<400> 72

```
Met Val Val Ala Met Asp Gln Arg Ser Asn Ala Asn Gly Asp Glu Arg
 1               5                  10                  15
Phe Asp Pro Ser Ala Gln Pro Pro Phe Lys Ile Gly Asp Ile Arg Ala
```

```
                20                  25                  30
Ala Ile Pro Lys His Cys Trp Val Lys Ser Pro Leu Arg Ser Met Ser
        35                  40                  45
Tyr Val Ala Arg Asp Ile Phe Ala Val Val Ala Leu Ala Val Ala Ala
    50                  55                  60
Val Tyr Phe Asp Ser Trp Phe Phe Trp Pro Leu Tyr Trp Ala Ala Gln
65                  70                  75                  80
Gly Thr Leu Phe Trp Ala Ile Phe Val Leu Gly His Asp Cys Gly His
                85                  90                  95
Gly Ser Phe Ser Asp Ile Pro Leu Leu Asn Thr Ala Val Gly His Ile
            100                 105                 110
Leu His Ser Phe Ile Leu Val Pro Tyr His Gly Trp Arg Ile Ser His
        115                 120                 125
Arg Thr His His Gln Asn His Gly His Val Glu Asn Asp Glu Ser Trp
    130                 135                 140
Val Pro Leu Pro Glu Lys Leu Tyr Lys Asn Leu Ser His Ser Thr Arg
145                 150                 155                 160
Met Leu Arg Tyr Thr Val Pro Leu Pro Met Leu Ala Tyr Pro Leu Tyr
                165                 170                 175
Leu Trp Tyr Arg Ser Pro Gly Lys Glu Gly Ser His Tyr Asn Pro Tyr
            180                 185                 190
Ser Ser Leu Phe Ala Pro Ser Glu Arg Lys Leu Ile Ala Thr Ser Thr
        195                 200                 205
Thr Cys Trp Ser Ile Met Leu Ala Thr Leu Val Tyr Leu Ser Phe Leu
    210                 215                 220
Val Gly Pro Val Thr Val Leu Lys Val Tyr Gly Val Pro Tyr Ile Ile
225                 230                 235                 240
Phe Val Met Trp Leu Asp Ala Val Thr Tyr Leu His His His Gly His
                245                 250                 255
Asp Asp Lys Leu Pro Trp Tyr Arg Gly Lys Glu Trp Ser Tyr Leu Arg
            260                 265                 270
Gly Gly Leu Thr Thr Ile Asp Arg Asp Tyr Gly Ile Phe Asn Asn Ile
        275                 280                 285
His His Asp Ile Gly Thr His Val Ile His His Leu Phe Pro Gln Ile
    290                 295                 300
Pro His Tyr His Leu Val Asp Ala Thr Lys Ser Ala Lys His Val Leu
305                 310                 315                 320
Gly Arg Tyr Tyr Arg Glu Pro Lys Thr Ser Gly Ala Ile Pro Ile His
                325                 330                 335
Leu Val Glu Ser Leu Val Ala Ser Ile Lys Lys Asp His Tyr Val Ser
            340                 345                 350
Asp Thr Gly Asp Ile Val Phe Tyr Glu Thr Asp Pro Asp Leu Tyr Val
        355                 360                 365
Tyr Ala Ser Asp Lys Ser Lys Ile Asn
    370                 375
```

<210> 73
<211> 380
<212> PRT
<213> Glycine soya

<400> 73

```
Met Val Lys Asp Thr Lys Pro Leu Ala Tyr Ala Ala Asn Asn Gly Tyr
 1               5                  10                  15
Gln Gln Lys Gly Ser Ser Phe Asp Phe Asp Pro Ser Ala Pro Pro Pro
            20                  25                  30
Phe Lys Ile Ala Glu Ile Arg Ala Ser Ile Pro Lys His Cys Trp Val
        35                  40                  45
```

```
Lys Asn Pro Trp Arg Ser Leu Ser Tyr Val Leu Arg Asp Val Leu Val
    50                  55                  60
Ile Ala Ala Leu Val Ala Ala Ala Ile His Phe Asp Asn Trp Leu Leu
65                  70                  75                  80
Trp Leu Ile Tyr Cys Pro Ile Gln Gly Thr Met Phe Trp Ala Leu Phe
                85                  90                  95
Val Leu Gly His Asp Cys Gly His Gly Ser Phe Ser Asp Ser Pro Leu
            100                 105                 110
Leu Asn Ser Leu Val Gly His Ile Leu His Ser Ser Ile Leu Val Pro
        115                 120                 125
Tyr His Gly Trp Arg Ile Ser His Arg Thr His His Gln Asn His Gly
    130                 135                 140
His Ile Glu Lys Asp Glu Ser Trp Val Pro Leu Thr Glu Lys Ile Tyr
145                 150                 155                 160
Lys Asn Leu Asp Ser Met Thr Arg Leu Ile Arg Phe Thr Val Pro Phe
                165                 170                 175
Pro Leu Phe Val Tyr Pro Ile Tyr Leu Phe Ser Arg Ser Pro Gly Lys
            180                 185                 190
Glu Gly Ser His Phe Asn Pro Tyr Ser Asn Leu Phe Pro Pro Ser Glu
        195                 200                 205
Arg Lys Gly Ile Ala Ile Ser Thr Leu Cys Trp Ala Thr Met Phe Ser
    210                 215                 220
Leu Leu Ile Tyr Leu Ser Phe Ile Thr Ser Pro Leu Leu Val Leu Lys
225                 230                 235                 240
Leu Tyr Gly Ile Pro Tyr Trp Ile Phe Val Met Trp Leu Asp Phe Val
                245                 250                 255
Thr Tyr Leu His His His Gly His His Gln Lys Leu Pro Trp Tyr Arg
            260                 265                 270
Gly Lys Glu Trp Ser Tyr Leu Arg Gly Gly Leu Thr Thr Val Asp Arg
        275                 280                 285
Asp Tyr Gly Trp Ile Tyr Asn Ile His His Asp Ile Gly Thr His Val
    290                 295                 300
Ile His His Leu Phe Pro Gln Ile Pro His Tyr His Leu Val Glu Ala
305                 310                 315                 320
Thr Gln Ala Ala Lys Pro Val Leu Gly Asp Tyr Tyr Arg Glu Pro Glu
                325                 330                 335
Arg Ser Ala Pro Leu Pro Phe His Leu Ile Lys Tyr Leu Ile Gln Ser
            340                 345                 350
Met Arg Gln Asp His Phe Val Ser Asp Thr Gly Asp Val Val Tyr Tyr
        355                 360                 365
Gln Thr Asp Ser Leu Leu Leu His Ser Gln Arg Asp
    370                 375                 380


<210> 74
<211> 519
<212> PRT
<213> Thraustochytrium aureum

<400> 74
Met Thr Val Gly Tyr Asp Glu Glu Ile Pro Phe Glu Gln Val Arg Ala
 1               5                  10                  15
His Asn Lys Pro Asp Asp Ala Trp Cys Ala Ile His Gly His Val Tyr
            20                  25                  30
Asp Val Thr Lys Phe Ala Ser Val His Pro Gly Gly Asp Ile Ile Leu
        35                  40                  45
Leu Ala Ala Gly Lys Glu Ala Thr Val Leu Tyr Glu Thr Tyr His Val
    50                  55                  60
Arg Gly Val Ser Asp Ala Val Leu Arg Lys Tyr Arg Ile Gly Lys Leu
```

65 70 75 80
Pro Asp Gly Gln Gly Gly Ala Asn Glu Lys Glu Lys Arg Thr Leu Ser
85 90 95
Gly Leu Ser Ser Ala Ser Tyr Tyr Thr Trp Asn Ser Asp Phe Tyr Arg
100 105 110
Val Met Arg Glu Arg Val Val Ala Arg Leu Lys Glu Arg Gly Lys Ala
115 120 125
Arg Arg Gly Gly Tyr Glu Leu Trp Ile Lys Ala Phe Leu Leu Leu Val
130 135 140
Gly Phe Trp Ser Ser Leu Tyr Trp Met Cys Thr Leu Asp Pro Ser Phe
145 150 155 160
Gly Ala Ile Leu Ala Ala Met Ser Leu Gly Val Phe Ala Ala Phe Val
165 170 175
Gly Thr Cys Ile Gln His Asp Gly Asn His Gly Ala Phe Ala Gln Ser
180 185 190
Arg Trp Val Asn Lys Val Ala Gly Trp Thr Leu Asp Met Ile Gly Ala
195 200 205
Ser Gly Met Thr Trp Glu Phe Gln His Val Leu Gly His His Pro Tyr
210 215 220
Thr Asn Leu Ile Glu Glu Glu Asn Gly Leu Gln Lys Val Ser Gly Lys
225 230 235 240
Lys Met Asp Thr Lys Leu Ala Asp Gln Glu Ser Asp Pro Asp Val Phe
245 250 255
Ser Thr Tyr Pro Met Met Arg Leu His Pro Trp His Gln Lys Arg Trp
260 265 270
Tyr His Arg Phe Gln His Ile Tyr Gly Pro Phe Ile Phe Gly Phe Met
275 280 285
Thr Ile Asn Lys Val Val Thr Gln Asp Val Gly Val Val Leu Arg Lys
290 295 300
Arg Leu Phe Gln Ile Asp Ala Glu Cys Arg Tyr Ala Ser Pro Met Tyr
305 310 315 320
Val Ala Arg Phe Trp Ile Met Lys Ala Leu Thr Val Leu Tyr Met Val
325 330 335
Ala Leu Pro Cys Tyr Met Gln Gly Pro Trp His Gly Leu Lys Leu Phe
340 345 350
Ala Ile Ala His Phe Thr Cys Gly Glu Val Leu Ala Thr Met Phe Ile
355 360 365
Val Asn His Ile Ile Glu Gly Val Ser Tyr Ala Ser Lys Asp Ala Val
370 375 380
Lys Gly Thr Met Ala Pro Pro Lys Thr Met His Gly Val Thr Pro Met
385 390 395 400
Asn Asn Thr Arg Lys Glu Val Glu Ala Glu Ala Ser Lys Ser Gly Ala
405 410 415
Val Val Lys Ser Val Pro Leu Asp Asp Trp Ala Val Val Gln Cys Gln
420 425 430
Thr Ser Val Asn Trp Ser Val Gly Ser Trp Phe Trp Asn His Phe Ser
435 440 445
Gly Gly Leu Asn His Gln Ile Glu His His Leu Phe Pro Gly Leu Ser
450 455 460
His Glu Thr Tyr Tyr His Ile Gln Asp Val Phe Gln Ser Thr Cys Ala
465 470 475 480
Glu Tyr Gly Val Pro Tyr Gln His Glu Pro Ser Leu Trp Thr Ala Tyr
485 490 495
Trp Lys Met Leu Glu His Leu Arg Gln Leu Gly Asn Glu Glu Thr His
500 505 510
Glu Ser Trp Gln Arg Ala Ala
515

<210> 75
<211> 541
<212> PRT
<213> Euglena gracilis

<400> 75

```
Met Leu Val Leu Phe Gly Asn Phe Tyr Val Lys Gln Tyr Ser Gln Lys
 1               5                  10                  15
Asn Gly Lys Pro Glu Asn Gly Ala Thr Pro Glu Asn Gly Ala Lys Pro
            20                  25                  30
Gln Pro Cys Glu Asn Gly Thr Val Glu Lys Arg Glu Asn Asp Thr Ala
        35                  40                  45
Asn Val Arg Pro Thr Arg Pro Ala Gly Pro Pro Pro Ala Thr Tyr Tyr
    50                  55                  60
Asp Ser Leu Ala Val Ser Gly Gln Gly Lys Glu Arg Leu Phe Thr Thr
65                  70                  75                  80
Asp Glu Val Arg Arg His Ile Leu Pro Thr Asp Gly Trp Leu Thr Cys
                85                  90                  95
His Glu Gly Val Tyr Asp Val Thr Asp Phe Leu Ala Lys His Pro Gly
            100                 105                 110
Gly Gly Val Ile Thr Leu Gly Leu Gly Arg Asp Cys Thr Ile Leu Ile
        115                 120                 125
Glu Ser Tyr His Pro Ala Gly Arg Pro Asp Lys Val Met Glu Lys Tyr
    130                 135                 140
Arg Ile Gly Thr Leu Gln Asp Pro Lys Thr Phe Tyr Ala Trp Gly Glu
145                 150                 155                 160
Ser Asp Phe Tyr Pro Glu Leu Lys Arg Arg Ala Leu Ala Arg Leu Lys
                165                 170                 175
Glu Ala Gly Gln Ala Arg Arg Gly Gly Leu Gly Val Lys Ala Leu Leu
            180                 185                 190
Val Leu Thr Leu Phe Phe Val Ser Trp Tyr Met Trp Val Ala His Lys
        195                 200                 205
Ser Phe Leu Trp Ala Ala Val Trp Gly Phe Ala Gly Ser His Val Gly
    210                 215                 220
Leu Ser Ile Gln His Asp Gly Asn His Gly Ala Phe Ser Arg Asn Thr
225                 230                 235                 240
Leu Val Asn Arg Leu Ala Gly Trp Gly Met Asp Leu Ile Gly Ala Ser
                245                 250                 255
Ser Thr Val Trp Glu Tyr Gln His Val Ile Gly His His Gln Tyr Thr
            260                 265                 270
Asn Leu Val Ser Asp Thr Leu Phe Ser Leu Pro Glu Asn Asp Pro Asp
        275                 280                 285
Val Phe Ser Ser Tyr Pro Leu Met Arg Met His Pro Asp Thr Ala Trp
    290                 295                 300
Gln Pro His His Arg Phe Gln His Leu Phe Ala Phe Pro Leu Phe Ala
305                 310                 315                 320
Leu Met Thr Ile Ser Lys Val Leu Thr Ser Asp Phe Ala Val Cys Leu
                325                 330                 335
Ser Met Lys Lys Gly Ser Ile Asp Cys Ser Ser Arg Leu Val Pro Leu
            340                 345                 350
Glu Gly Gln Leu Leu Phe Trp Gly Ala Lys Leu Ala Asn Phe Leu Leu
        355                 360                 365
Gln Ile Val Leu Pro Cys Tyr Leu His Gly Thr Ala Met Gly Leu Ala
    370                 375                 380
Leu Phe Ser Val Ala His Leu Val Ser Gly Glu Tyr Leu Ala Ile Cys
385                 390                 395                 400
Phe Ile Ile Asn His Ile Ser Glu Ser Cys Glu Phe Met Asn Thr Ser
                405                 410                 415
Phe Gln Thr Ala Ala Arg Arg Thr Glu Met Leu Gln Ala Ala His Gln
```

420 425 430
Ala Ala Glu Ala Lys Lys Val Lys Pro Thr Pro Pro Pro Asn Asp Trp
435 440 445
Ala Val Thr Gln Val Gln Cys Cys Val Asn Trp Arg Ser Gly Gly Val
450 455 460
Leu Ala Asn His Leu Ser Gly Gly Leu Asn His Gln Ile Glu His His
465 470 475 480
Leu Phe Pro Ser Ile Ser His Ala Asn Tyr Pro Thr Ile Ala Pro Val
485 490 495
Val Lys Glu Val Cys Glu Glu Tyr Gly Leu Pro Tyr Lys Asn Tyr Val
500 505 510
Thr Phe Trp Asp Ala Val Cys Gly Met Val Gln His Leu Arg Leu Met
515 520 525
Gly Ala Pro Pro Val Pro Thr Asn Gly Asp Lys Lys Ser
530 535 540

<210> 76
<211> 433
<212> PRT
<213> Isochrysis galbana

<400> 76
Met Cys Asn Ala Ala Gln Val Glu Thr Gln Ala Leu Arg Ala Lys Glu
1 5 10 15
Ala Ala Lys Pro Thr Trp Thr Lys Ile His Gly Arg Thr Val Asp Val
20 25 30
Glu Thr Phe Arg His Pro Gly Gly Asn Ile Leu Asp Leu Phe Leu Gly
35 40 45
Met Asp Ala Thr Thr Ala Phe Glu Thr Phe His Gly His His Lys Gly
50 55 60
Ala Trp Lys Met Leu Lys Thr Leu Pro Glu Lys Glu Val Ala Ala Ala
65 70 75 80
Asp Ile Pro Ala Gln Lys Glu Glu His Val Ala Glu Met Thr Arg Leu
85 90 95
Met Ala Ser Trp Arg Glu Arg Gly Leu Phe Lys Pro Arg Pro Val Ala
100 105 110
Ser Ser Ile Tyr Gly Leu Cys Val Ile Phe Ala Ile Ala Ala Ser Val
115 120 125
Ala Cys Ala Pro Tyr Ala Pro Val Leu Ala Gly Ile Ala Val Gly Thr
130 135 140
Cys Trp Ala Gln Cys Gly Phe Leu Gln His Met Gly Gly His Arg Glu
145 150 155 160
Trp Gly Arg Thr Trp Ser Phe Ala Phe Gln His Leu Phe Glu Gly Leu
165 170 175
Leu Lys Gly Gly Ser Ala Ser Trp Trp Arg Asn Arg His Asn Lys His
180 185 190
His Ala Lys Thr Asn Val Leu Gly Glu Asp Gly Asp Leu Arg Thr Thr
195 200 205
Pro Phe Phe Ala Trp Asp Pro Thr Leu Ala Lys Lys Val Pro Asp Trp
210 215 220
Ser Leu Arg Thr Gln Ala Phe Thr Phe Leu Pro Ala Leu Gly Ala Tyr
225 230 235 240
Val Phe Val Phe Ala Phe Thr Val Arg Lys Tyr Ser Val Val Lys Arg
245 250 255
Leu Trp His Glu Val Ala Leu Met Val Ala His Tyr Ala Leu Phe Ser
260 265 270
Trp Ala Leu Ser Ala Ala Gly Ala Ser Leu Ser Ser Gly Leu Thr Phe
275 280 285

Tyr Cys Thr Gly Tyr Ala Trp Gln Gly Ile Tyr Leu Gly Phe Phe Phe
290 295 300
Gly Leu Ser His Phe Ala Val Glu Arg Val Pro Ser Thr Ala Thr Trp
305 310 315 320
Leu Glu Ser Thr Met Met Gly Thr Val Asp Trp Gly Gly Ser Ser Ala
325 330 335
Phe Cys Gly Tyr Leu Ser Gly Phe Leu Asn Ile Gln Ile Glu His His
340 345 350
Met Ala Pro Gln Met Pro Met Glu Asn Leu Arg Gln Ile Arg Ala Asp
355 360 365
Cys Lys Ala Ala Ala His Lys Phe Gly Leu Pro Tyr Arg Glu Leu Thr
370 375 380
Phe Val Ala Ala Thr Lys Leu Met Met Ser Gly Leu Tyr Arg Thr Gly
385 390 395 400
Lys Asp Glu Leu Lys Leu Arg Ala Asp Arg Arg Lys Phe Thr Arg Ala
405 410 415
Gln Ala Tyr Met Gly Ala Ala Ser Ala Leu Val Asp Thr Leu Lys Ala
420 425 430
Asp

<210> 77
<211> 509
<212> PRT
<213> Schizochytrium aggregatum

<400> 77
Met Thr Val Gly Gly Asp Glu Val Tyr Ser Met Ala Gln Val Arg Asp
1 5 10 15
His Asn Thr Pro Asp Asp Ala Trp Cys Ala Ile His Gly Glu Val Tyr
20 25 30
Glu Leu Thr Lys Phe Ala Arg Thr His Pro Gly Gly Asp Ile Ile Leu
35 40 45
Leu Ala Ala Gly Lys Glu Ala Thr Ile Leu Phe Glu Thr Tyr His Val
50 55 60
Arg Pro Ile Ser Asp Ala Val Leu Arg Lys Tyr Arg Ile Gly Lys Leu
65 70 75 80
Ala Ala Ala Gly Lys Asp Glu Pro Ala Asn Asp Ser Thr Tyr Tyr Ser
85 90 95
Trp Asp Ser Asp Phe Tyr Lys Val Leu Arg Gln Arg Val Val Ala Arg
100 105 110
Leu Glu Glu Arg Lys Ile Ala Arg Arg Gly Gly Pro Glu Ile Trp Ile
115 120 125
Lys Ala Ala Ile Leu Val Ser Gly Phe Trp Ser Met Leu Tyr Leu Met
130 135 140
Cys Thr Leu Asp Pro Asn Arg Gly Ala Ile Leu Ala Ala Ile Ala Leu
145 150 155 160
Gly Ile Val Ala Ala Phe Val Gly Thr Cys Ile Gln His Asp Gly Asn
165 170 175
His Gly Ala Phe Ala Phe Ser Pro Phe Met Asn Lys Leu Ser Gly Trp
180 185 190
Thr Leu Asp Met Ile Gly Ala Ser Ala Met Thr Trp Glu Met Gln His
195 200 205
Val Leu Gly His His Pro Tyr Thr Asn Leu Ile Glu Met Glu Asn Gly
210 215 220
Thr Gln Lys Val Thr His Ala Asp Val Asp Pro Lys Lys Ala Asp Gln
225 230 235 240
Glu Ser Asp Pro Asp Val Phe Ser Thr Tyr Pro Met Leu Arg Leu His

245 250 255
Pro Trp His Arg Lys Arg Phe Tyr His Arg Phe Gln His Leu Tyr Ala
260 265 270
Pro Leu Leu Phe Gly Phe Met Thr Ile Asn Lys Val Ile Thr Gln Asp
275 280 285
Val Gly Val Val Leu Ser Lys Arg Leu Phe Gln Ile Asp Ala Asn Cys
290 295 300
Arg Tyr Ala Ser Lys Ser Tyr Val Ala Arg Phe Trp Ile Met Lys Leu
305 310 315 320
Leu Thr Val Leu Tyr Met Val Ala Leu Pro Val Tyr Thr Gln Gly Leu
325 330 335
Val Asp Gly Leu Lys Leu Phe Phe Ile Ala His Phe Ser Cys Gly Glu
340 345 350
Leu Leu Ala Thr Met Phe Ile Val Asn His Ile Ile Glu Gly Val Ser
355 360 365
Tyr Ala Ser Lys Asp Ser Val Lys Gly Thr Met Ala Pro Pro Arg Thr
370 375 380
Val His Gly Val Thr Pro Met His Asp Thr Arg Asp Ala Leu Gly Lys
385 390 395 400
Glu Lys Ala Ala Thr Lys His Val Pro Leu Asn Asp Trp Ala Ala Val
405 410 415
Gln Cys Gln Thr Ser Val Asn Trp Ser Ile Gly Ser Trp Phe Trp Asn
420 425 430
His Phe Ser Gly Gly Leu Asn His Gln Ile Glu His His Leu Phe Pro
435 440 445
Gly Leu Thr His Thr Thr Tyr Val Tyr Ile Gln Asp Val Val Gln Ala
450 455 460
Thr Cys Ala Glu Tyr Gly Val Pro Tyr Gln Ser Glu Gln Ser Leu Phe
465 470 475 480
Ser Ala Tyr Phe Lys Met Leu Ser His Leu Arg Ala Leu Gly Asn Glu
485 490 495
Pro Met Pro Ser Trp Glu Lys Asp His Pro Lys Ser Lys
500 505

<210> 78
<211> 263
<212> PRT
<213> Isochrysis galbana

<400> 78
Met Ala Leu Ala Asn Asp Ala Gly Glu Arg Ile Trp Ala Ala Val Thr
1 5 10 15
Asp Pro Glu Ile Leu Ile Gly Thr Phe Ser Tyr Leu Leu Leu Lys Pro
20 25 30
Leu Leu Arg Asn Ser Gly Leu Val Asp Glu Lys Lys Gly Ala Tyr Arg
35 40 45
Thr Ser Met Ile Trp Tyr Asn Val Leu Leu Ala Leu Phe Ser Ala Leu
50 55 60
Ser Phe Tyr Val Thr Ala Thr Ala Leu Gly Trp Asp Tyr Gly Thr Gly
65 70 75 80
Ala Trp Leu Arg Arg Gln Thr Gly Asp Thr Pro Gln Pro Leu Phe Gln
85 90 95
Cys Pro Ser Pro Val Trp Asp Ser Lys Leu Phe Thr Trp Thr Ala Lys
100 105 110
Ala Phe Tyr Tyr Ser Lys Tyr Val Glu Tyr Leu Asp Thr Ala Trp Leu
115 120 125
Val Leu Lys Gly Lys Arg Val Ser Phe Leu Gln Ala Phe His His Phe
130 135 140

```
Gly Ala Pro Trp Asp Val Tyr Leu Gly Ile Arg Leu His Asn Glu Gly
145                 150                 155                 160
Val Trp Ile Phe Met Phe Phe Asn Ser Phe Ile His Thr Ile Met Tyr
                165                 170                 175
Thr Tyr Tyr Gly Leu Thr Ala Ala Gly Tyr Lys Phe Lys Ala Lys Pro
            180                 185                 190
Leu Ile Thr Ala Met Gln Ile Cys Gln Phe Val Gly Gly Phe Leu Leu
        195                 200                 205
Val Trp Asp Tyr Ile Asn Val Pro Cys Phe Asn Ser Asp Lys Gly Lys
    210                 215                 220
Leu Phe Ser Trp Ala Phe Asn Tyr Ala Tyr Val Gly Ser Val Phe Leu
225                 230                 235                 240
Leu Phe Cys His Phe Phe Tyr Gln Asp Asn Leu Ala Thr Lys Lys Ser
                245                 250                 255
Ala Lys Ala Gly Lys Gln Leu
            260
```

<210> 79
<211> 419
<212> PRT
<213> Euglena gracilis

<400> 79

```
Met Lys Ser Lys Arg Gln Ala Leu Ser Pro Leu Gln Leu Met Glu Gln
 1               5                  10                  15
Thr Tyr Asp Val Val Asn Phe His Pro Gly Gly Ala Glu Ile Ile Glu
            20                  25                  30
Asn Tyr Gln Gly Arg Asp Ala Thr Asp Ala Phe Met Val Met His Phe
        35                  40                  45
Gln Glu Ala Phe Asp Lys Leu Lys Arg Met Pro Lys Ile Asn Pro Ser
    50                  55                  60
Phe Glu Leu Pro Pro Gln Ala Ala Val Asn Glu Ala Gln Glu Asp Phe
65                  70                  75                  80
Arg Lys Leu Arg Glu Glu Leu Ile Ala Thr Gly Met Phe Asp Ala Ser
                85                  90                  95
Pro Leu Trp Tyr Ser Tyr Lys Ile Ser Thr Thr Leu Gly Leu Gly Val
            100                 105                 110
Leu Gly Tyr Phe Leu Met Val Gln Tyr Gln Met Tyr Phe Ile Gly Ala
        115                 120                 125
Val Leu Leu Gly Met His Tyr Gln Gln Met Gly Trp Leu Ser His Asp
    130                 135                 140
Ile Cys His His Gln Thr Phe Lys Asn Arg Asn Trp Asn Asn Leu Val
145                 150                 155                 160
Gly Leu Val Phe Gly Asn Gly Leu Gln Gly Phe Ser Val Thr Cys Trp
                165                 170                 175
Lys Asp Arg His Asn Ala His His Ser Ala Thr Asn Val Gln Gly His
            180                 185                 190
Asp Pro Asp Ile Asp Asn Leu Pro Pro Leu Ala Trp Ser Glu Asp Asp
        195                 200                 205
Val Thr Arg Ala Ser Pro Ile Ser Arg Lys Leu Ile Gln Phe Gln Gln
    210                 215                 220
Tyr Tyr Phe Leu Val Ile Cys Ile Leu Leu Arg Phe Ile Trp Cys Phe
225                 230                 235                 240
Gln Cys Val Leu Thr Val Arg Ser Leu Lys Asp Arg Asp Asn Gln Phe
                245                 250                 255
Tyr Arg Ser Gln Tyr Lys Lys Glu Ala Ile Gly Leu Ala Leu His Trp
            260                 265                 270
Thr Leu Lys Ala Leu Phe His Leu Phe Phe Met Pro Ser Ile Leu Thr
```

```
         275                 280                 285
Ser Leu Leu Val Phe Phe Val Ser Glu Leu Val Gly Gly Phe Gly Ile
     290                 295                 300
Ala Ile Val Val Phe Met Asn His Tyr Pro Leu Glu Lys Ile Gly Asp
305                 310                 315                 320
Pro Val Trp Asp Gly His Gly Phe Ser Val Gly Gln Ile His Glu Thr
                325                 330                 335
Met Asn Ile Arg Arg Gly Ile Ile Thr Asp Trp Phe Phe Gly Gly Leu
            340                 345                 350
Asn Tyr Gln Ile Glu His His Leu Trp Pro Thr Leu Pro Arg His Asn
         355                 360                 365
Leu Thr Ala Val Ser Tyr Gln Val Glu Gln Leu Cys Gln Lys His Asn
     370                 375                 380
Leu Pro Tyr Arg Asn Pro Leu Pro His Glu Gly Leu Val Ile Leu Leu
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Lys Ala Leu
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<210> 80
<211> 2025
<212> DNA
<213> Sordaria macrospora

<400> 80

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<210> 81
<211> 674
<212> PRT
<213> Sordaria macrospora

<400> 81

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        35                  40                  45
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    50                  55                  60
Gln Pro Arg Ala Val Gln Gly Met Leu Asp Phe Asp Phe Ile Cys Lys
65                  70                  75                  80
Arg Ser Thr Pro Ser Val Ala Gly Ile Ile Tyr Thr Phe Gly Gly Gln
                85                  90                  95
Phe Val Ser Lys Met Tyr Trp Gly Thr Ser Glu Thr Leu Leu Pro Val
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Tyr Gln Glu Val Gln Lys Ala Ile Ala Lys His Pro Asp Val Asp Val
        115                 120                 125
Val Val Asn Phe Ala Ser Ser Arg Ser Val Tyr Ser Ser Thr Met Glu
    130                 135                 140
Leu Met Glu His Pro Gln Ile Lys Thr Ile Ala Ile Ile Ala Glu Gly
145                 150                 155                 160
Val Pro Glu Arg Arg Ala Arg Glu Ile Ala Tyr Val Ala Lys Lys Lys
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Gly Ile Thr Ile Ile Gly Pro Ala Thr Val Gly Gly Ile Lys Pro Gly
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Cys Phe Lys Ile Gly Asn Thr Gly Gly Met Met Asp Asn Ile Val Ala
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Ser Lys Leu Tyr Arg Lys Gly Ser Val Gly Tyr Val Ser Lys Ser Gly
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Gly Met Ser Asn Glu Leu Asn Asn Ile Ile Ser Gln Thr Thr Asp Gly
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Val Tyr Glu Gly Val Ala Ile Gly Gly Asp Arg Tyr Pro Gly Thr Thr
                245                 250                 255
Phe Ile Asp His Leu Leu Arg Tyr Gln Ala Asp Pro Ala Cys Lys Ile
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Leu Val Leu Leu Gly Glu Val Gly Gly Val Glu Glu Tyr Lys Val Ile
        275                 280                 285
Glu Ala Val Lys Gln Gly Ile Ile Thr Lys Pro Ile Val Ala Trp Ala
    290                 295                 300
Ile Gly Thr Cys Ala Ser Met Phe Lys Thr Glu Val Gln Phe Gly His
305                 310                 315                 320
Ala Gly Ala Phe Ala Asn Ser Gln Leu Glu Thr Ala Ala Thr Lys Asn
                325                 330                 335
Lys Ser Met Arg Glu Ala Gly Phe Tyr Val Pro Asp Thr Phe Glu Asp
            340                 345                 350
Met Pro Ala Leu Leu Lys Gln Val Tyr Asp Lys Leu Val Ala Asp Gly
        355                 360                 365
Thr Ile Val Pro Ala Pro Glu Pro Val Val Pro Lys Ile Pro Ile Asp
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Tyr Ser Trp Ala Gln Glu Leu Gly Leu Ile Arg Lys Pro Ala Ala Phe
385                 390                 395                 400
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Ile Ser Thr Ile Ser Asp Asp Arg Gly Gln Glu Leu Leu Tyr Ala Gly
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Met Pro Ile Ser Asp Val Phe Arg Glu Glu Ile Gly Ile Gly Gly Val
420 425 430
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435 440 445
Phe Leu Glu Met Val Leu Met Leu Thr Ala Asp His Gly Pro Ala Val
450 455 460
Ser Gly Ala Met Asn Thr Ile Ile Thr Thr Arg Ala Gly Lys Asp Leu
465 470 475 480
Ile Ser Ser Leu Val Ala Gly Leu Leu Thr Ile Gly Ser Arg Phe Gly
485 490 495
Gly Ala Leu Asp Gly Ala Ala Glu Glu Phe Thr Lys Ala Phe Asp Lys
500 505 510
Gly Leu Ser Pro Arg Glu Phe Val Asp Thr Met Arg Lys Gln Asn Lys
515 520 525
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530 535 540
Leu Arg Val Glu Leu Val Lys Glu Tyr Val Lys Ala Lys Phe Pro Ser
545 550 555 560
Ser Lys Leu Leu Asp Tyr Ala Leu Ala Val Glu Thr Val Thr Thr Ser
565 570 575
Lys Lys Asp Asn Leu Ile Leu Asn Val Asp Gly Cys Ile Ala Val Cys
580 585 590
Phe Val Asp Leu Leu Arg Asn Cys Gly Ala Phe Ser Thr Glu Glu Ala
595 600 605
Glu Asp Tyr Leu Ser Met Gly Val Leu Asn Gly Leu Phe Val Leu Gly
610 615 620
Arg Ser Ile Gly Leu Ile Ala His Tyr Leu Asp Gln Lys Arg Leu Arg
625 630 635 640
Thr Gly Leu Tyr Arg His Pro Trp Asp Asp Ile Thr Tyr Leu Leu Pro
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<210> 82
<211> 1446
<212> DNA
<213> Sordaria macrospora

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cttcgccagt tcctcgtcga gccgttcgtg ccccaccccc aggaaaccga atattatatc 420
aacggcgact ggattctctt ctaccacgag ggcggtgttg atgtcggtga cgtcgatgcg 480
aaggccgaga agatcctaat ccccgttgac ctttcacaat atccttccaa cgaggaactt 540
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gcctaa 1446

<210> 83
<211> 481
<212> PRT
<213> Sordaria macrospora

<400> 83

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			20					25				30			
Pro	Thr	Lys	His	Asn	Pro	Pro	Pro	Arg	Leu	Ala	Ser	Leu	His	Phe	Ala
		35					40					45			
Glu	Asp	Ala	Asp	Val	Asn	Gly	Val	Leu	Ser	Gln	Ala	Glu	Val	Thr	Tyr
	50					55					60				
Pro	Trp	Leu	Leu	Gln	Asp	Gly	Ala	Arg	Phe	Val	Ala	Lys	Pro	Asp	Gln
65					70					75					80
Leu	Ile	Lys	Arg	Arg	Gly	Lys	Ser	Gly	Leu	Leu	Ala	Leu	Asn	Lys	Thr
				85					90					95	
Trp	Pro	Glu	Ala	Lys	Ala	Trp	Ile	Ala	Glu	Arg	Ala	Gly	Lys	Ala	Gln
			100					105					110		
Lys	Val	Glu	His	Thr	Glu	Gly	Val	Leu	Arg	Gln	Phe	Leu	Val	Glu	Pro
		115					120					125			
Phe	Val	Pro	His	Pro	Gln	Glu	Thr	Glu	Tyr	Tyr	Ile	Asn	Gly	Asp	Trp
	130					135					140				
Ile	Leu	Phe	Tyr	His	Glu	Gly	Gly	Val	Asp	Val	Gly	Asp	Val	Asp	Ala
145					150					155					160
Lys	Ala	Glu	Lys	Ile	Leu	Ile	Pro	Val	Asp	Leu	Ser	Gln	Tyr	Pro	Ser
				165					170					175	
Asn	Glu	Glu	Leu	Ala	Ser	Thr	Leu	Leu	Lys	His	Val	Pro	Lys	Gly	Ile
			180					185					190		
His	Asn	Val	Leu	Val	Asp	Phe	Ile	Ala	Arg	Leu	Tyr	Ala	Val	Tyr	Val
		195					200					205			
Asp	Cys	Gln	Phe	Thr	Tyr	Leu	Glu	Ile	Asn	Pro	Leu	Val	Val	Ile	Pro
	210					215					220				
Asn	Glu	Asp	Ala	Thr	Ser	Ala	Glu	Val	His	Phe	Leu	Asp	Leu	Ala	Ala
225					230					235					240
Lys	Leu	Asp	Gln	Thr	Ala	Asp	Phe	Glu	Cys	Gly	Asn	Lys	Trp	Ala	Ile
				245					250					255	
Ala	Arg	Ser	Pro	Ala	Ala	Leu	Gly	Ile	Val	Ala	Gln	Ser	Ser	Asn	Gly
			260					265					270		
Gly	Val	Asn	Ile	Asp	Ala	Gly	Pro	Pro	Ile	Glu	Phe	Pro	Ala	Pro	Phe
		275					280					285			
Gly	Arg	Glu	Leu	Thr	Lys	Glu	Glu	Ala	Tyr	Ile	Ala	Glu	Leu	Asp	Ala
	290					295					300				
Lys	Thr	Gly	Ala	Ser	Leu	Lys	Leu	Thr	Val	Leu	Asn	Pro	Asn	Gly	Arg
305					310					315					320
Ile	Trp	Thr	Leu	Val	Ala	Gly	Gly	Gly	Ala	Ser	Val	Val	Tyr	Ala	Asp
				325					330					335	

Ala Ile Ala Ser Ala Gly Phe Ala Asp Glu Leu Ala Asn Tyr Gly Glu
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Tyr Ser Gly Ala Pro Thr Glu Ser Gln Thr Tyr His Tyr Ala Arg Thr
355 360 365
Val Leu Asp Leu Met Leu Arg Ala Pro Val Ser Glu Gln Gly Lys Val
370 375 380
Leu Phe Ile Gly Gly Gly Ile Ala Asn Phe Thr Asn Val Ala Ser Thr
385 390 395 400
Phe Lys Gly Val Ile Lys Ala Leu Arg Glu Tyr Gly Lys Ala Leu Ile
405 410 415
Glu His Asn Thr Gln Ile Trp Val Arg Arg Ala Gly Pro Asn Tyr Gln
420 425 430
Glu Gly Leu Lys Asn Leu Lys Ala Ala Thr Gln Glu Leu Gly Leu Asn
435 440 445
Ala Lys Ile Phe Gly Pro Glu Met His Val Ser Gly Ile Val Pro Leu
450 455 460
Ala Leu Val Pro Gly Lys Trp Glu Glu Ser Gly Ala Val Glu Phe Gln
465 470 475 480
Ala

<210> 84
<211> 1560
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<221> CDS
<222> (0)...(0)
<223> synthetic nucleotide sequence encoding a delta-4 desaturase, codon-optimized for expression in S. cerevisiae

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acctatcatg taagaggcgt ttcagatgcc gttttaagaa agtatcgtat cggtaaactt 240
ccagacggcc aaggtggagc aaacgagaag gaaaaaagaa cgcttagtgg attatcgtca 300
gctagctact atacttggaa ctcggacttc tatcgtgtta tgagagagag agtagttgct 360
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caccatccat acacgaattt aattgaagag gaaaatggtt tgcaaaaagt tagtggcaaa 720
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gaggttttgg ctacaatgtt tatcgttaat catataattg aaggtgtatc ttatgcctcg 1140
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cccggtctgt ctcatgaaac ttattaccat attcaagatg tctttcaatc aacttgcgca 1440

gaatacggtg tgccctatca acacgaaccg agcttatgga ctgcttactg gaaaatgtta 1500
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<210> 85
<211> 792
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<221> CDS
<222> (0)...(0)
<223> synthetic nucleotide sequence encoding a delta-9 elongase, codon-optimized for expression in S. cerevisiae

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<210> 86
<211> 1260
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<221> CDS
<222> (0)...(0)
<223> synthetic nucleotide sequence encoding a delta-8 desaturase, codon-optimized for expression in S. cerevisiae

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attaacccct cctttgagtt gcctccacaa gctgccgtta atgaagctca ggaagacttt 240
aggaagttga gagaggaatt gattgcaacc ggcatgttcg atgctagccc attatggtat 300
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ccattggctt ggtcagaaga tgacgtcact agagcatctc ctatctctcg taagctgata 660
caatttcagc aatattactt tctggtgatt tgtatcttgc taagatttat ctggtgtttc 720
cagtgtgtct tgaccgtgag aagtcttaaa gacagagata atcaatttta ccgttctcaa 780
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<210> 87
<211> 1260
<212> DNA
<213> Saccharomyces kluyveri

<400> 87
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agaatgttca gaaacaccaa tggcgttggc gtcaagccgg aagatggttc cagtcaatga 1260

<210> 88
<211> 419
<212> PRT
<213> Saccharomyces kluyveri

<400> 88

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 1               5                  10                  15
Lys Ala Val Ser Ser Thr Ala Thr Thr Val Val Gln Pro Lys Thr Ala
            20                  25                  30
Ile Asp Thr Asn Gly Asn Val Phe Lys Val Pro Asp Tyr Thr Ile Lys
        35                  40                  45
Asp Ile Leu Ser Ala Ile Pro Lys Glu Cys Tyr Lys Arg Asp Thr Leu
    50                  55                  60
Trp Ser Leu His Tyr Val Val Arg Asp Ile Ala Ala Ile Leu Val Ile
65                  70                  75                  80
Gly Tyr Leu Gly Thr Asn Tyr Ile Pro Val Leu Phe Pro Asn Ser Ala
                85                  90                  95
Leu Leu Arg Gly Ile Ala Tyr Ala Ile Gln Ser Tyr Leu Ile Gly Leu
            100                 105                 110
Phe Gly Phe Gly Leu Trp Ile Leu Ala His Glu Cys Gly His Ser Ala
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115 120 125
Phe Ser Glu Ser Asn Ala Val Asn Asp Thr Val Gly Trp Val Leu His
130 135 140
Ser Trp Trp Met Val Pro Tyr Phe Pro Trp Lys Phe Ser His Ser Lys
145 150 155 160
His His Lys Ala Thr Gly His Met Thr Arg Asp Met Val Phe Ile Pro
165 170 175
Tyr Thr Lys Asp Glu Phe Ile Thr Met Lys Lys Lys Ser Lys Phe Ala
180 185 190
Glu Ile Thr Glu Glu Ala Pro Val Met Thr Leu Phe Asn Leu Ile Ala
195 200 205
Gln Gln Val Gly Gly Leu Gln Leu Tyr Leu Ala Thr Asn Ala Thr Gly
210 215 220
Gln Pro Tyr Pro Gly Val Lys Lys Phe Phe Lys Ser His Tyr Trp Pro
225 230 235 240
Thr Ser Pro Val Phe Asp Ala Lys Asp Phe Trp Trp Ile Ile Met Ser
245 250 255
Asp Ile Gly Ile Val Ser Thr Leu Leu Ile Asn Tyr Leu Trp Tyr Arg
260 265 270
Ala Tyr Gly Ala His Val Val Leu Ile Asn Trp Phe Ile Pro Trp Leu
275 280 285
Trp Val Asn His Trp Leu Val Phe Val Thr Phe Leu Gln His Thr Asp
290 295 300
Pro Thr Met Pro His Tyr Asp Ala Glu Glu Trp Thr Phe Ala Lys Gly
305 310 315 320
Ala Ala Ala Thr Ile Asp Arg Asn Phe Gly Phe Val Gly Gln His Ile
325 330 335
Phe His Asp Ile Ile Glu Thr His Val Leu His His Tyr Cys Ser Arg
340 345 350
Ile Pro Phe Tyr Asn Ala Arg Lys Ala Thr Ser Ala Ile Lys Glu Val
355 360 365
Met Gly Gln His Tyr Arg Tyr Glu Gly Glu Asn Met Trp Lys Ser Leu
370 375 380
Trp Lys Val Ala Arg Ser Cys Gln Tyr Val Glu Gly Asp Asn Gly Val
385 390 395 400
Arg Met Phe Arg Asn Thr Asn Gly Val Gly Val Lys Pro Glu Asp Gly
405 410 415
Ser Ser Gln

<210> 89
<211> 1353
<212> DNA
<213> Mortierella alpina

<400> 89
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gtcatggtcg gcccctggat cttggctcat ggtaaggaaa cgaaaaatcc catgtgtatt 360
tctgtactac agaaggcgaa gtttgtacct gaaaagatca gcgtcgtccc ttgatttaga 420
atgtaactaa ccttgcaatc gtatgaccta aattttcttg tgtcaacgac agagtgcggc 480
cacggagctt tctcggatag caagacgatc aacaccatct ttggatgggt cctccactct 540
gctcttttgg tgccctacca ggcctgggct atgtcacact ccaagcatca caagggtact 600
ggatcgatga ccaaagatgt cgttttcatc cctgccactc gttcctacaa gggcctccca 660
gcactggaga agcctgccgt cgaagaggag gtttcggagc aggaacacca ccaccacgag 720

gagtccatct ttgccgaaac tcccatctac acgctcggag cgcttttgtt cgtcttgacc 780
ttcggatggc ccttgtactt gatcgtcaac ttttcaggac acgaggcccc tcactgggtc 840
aaccatttcc agactgtcgc tcctctctat gagcctcacc agcgcaagaa catcttctac 900
tccaactgcg gcattgtcgc catgggttcg atcttgactt acctttcgat ggtcttctcg 960
cccttgactg tcttcatgta ctatggcatc ccttacctcg gagtcaacgc ctggatcgtc 1020
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aacttccagc gcggtgctgc ctgcactatc gaccgatcct tcggtaccat cgtgaaccac 1140
ctgcaccacc acattggcga ctctcaccag tgccaccata tgttctcgca gatgcccttc 1200
tacaatgctg tggaggctac aaagtacttg aaggccaaac ttggcaagta ctacatattt 1260
gacgacacgc ccattgccaa agccctctac cgcaattgga gagagtgcaa attcgtggag 1320
gacgagggag atgtagtgtt ttacaagcat taa 1353

<210> 90
<211> 403
<212> PRT
<213> Mortierella alpina

<400> 90

Met	Ala	Pro	Pro	His	Val	Val	Asp	Glu	Gln	Val	Arg	Arg	Arg	Ile	Val
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Val	Glu	Asp	Glu	Ile	Lys	Ser	Lys	Lys	Gln	Phe	Glu	Arg	Asn	Tyr	Val
			20					25				30			
Pro	Met	Asp	Phe	Thr	Ile	Lys	Glu	Ile	Arg	Asp	Ala	Ile	Pro	Ala	His
		35					40					45			
Leu	Phe	Ile	Arg	Asp	Thr	Thr	Lys	Ser	Ile	Leu	His	Val	Val	Lys	Asp
	50					55					60				
Leu	Val	Thr	Ile	Ala	Ile	Val	Phe	Tyr	Cys	Ala	Thr	Phe	Ile	Glu	Thr
65					70					75					80
Leu	Pro	Ser	Leu	Ala	Leu	Arg	Val	Pro	Ala	Trp	Ile	Thr	Tyr	Trp	Ile
				85					90					95	
Ile	Gln	Gly	Thr	Val	Met	Val	Gly	Pro	Trp	Ile	Leu	Ala	His	Glu	Cys
			100					105					110		
Gly	His	Gly	Ala	Phe	Ser	Asp	Ser	Lys	Thr	Ile	Asn	Thr	Ile	Phe	Gly
		115					120					125			
Trp	Val	Leu	His	Ser	Ala	Leu	Leu	Val	Pro	Tyr	Gln	Ala	Trp	Ala	Met
	130					135					140				
Ser	His	Ser	Lys	His	His	Lys	Gly	Thr	Gly	Ser	Met	Thr	Lys	Asp	Val
145					150					155					160
Val	Phe	Ile	Pro	Ala	Thr	Arg	Ser	Tyr	Lys	Gly	Leu	Pro	Ala	Leu	Glu
				165					170					175	
Lys	Pro	Ala	Val	Glu	Glu	Glu	Val	Ser	Glu	Gln	Glu	His	His	His	His
			180					185					190		
Glu	Glu	Ser	Ile	Phe	Ala	Glu	Thr	Pro	Ile	Tyr	Thr	Leu	Gly	Ala	Leu
		195					200					205			
Leu	Phe	Val	Leu	Thr	Phe	Gly	Trp	Pro	Leu	Tyr	Leu	Ile	Val	Asn	Phe
	210					215					220				
Ser	Gly	His	Glu	Ala	Pro	His	Trp	Val	Asn	His	Phe	Gln	Thr	Val	Ala
225					230					235					240
Pro	Leu	Tyr	Glu	Pro	His	Gln	Arg	Lys	Asn	Ile	Phe	Tyr	Ser	Asn	Cys
				245					250					255	
Gly	Ile	Val	Ala	Met	Gly	Ser	Ile	Leu	Thr	Tyr	Leu	Ser	Met	Val	Phe
			260					265					270		
Ser	Pro	Leu	Thr	Val	Phe	Met	Tyr	Tyr	Gly	Ile	Pro	Tyr	Leu	Gly	Val
		275					280					285			
Asn	Ala	Trp	Ile	Val	Cys	Ile	Thr	Tyr	Leu	Gln	His	Thr	Asp	Pro	Lys
	290					295					300				
Val	Pro	His	Phe	Arg	Asp	Asn	Glu	Trp	Asn	Phe	Gln	Arg	Gly	Ala	Ala
305					310					315					320
Cys	Thr	Ile	Asp	Arg	Ser	Phe	Gly	Thr	Ile	Val	Asn	His	Leu	His	His

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                325                 330                 335
His Ile Gly Asp Ser His Gln Cys His His Met Phe Ser Gln Met Pro
            340                 345                 350
Phe Tyr Asn Ala Val Glu Ala Thr Lys Tyr Leu Lys Ala Lys Leu Gly
        355                 360                 365
Lys Tyr Tyr Ile Phe Asp Asp Thr Pro Ile Ala Lys Ala Leu Tyr Arg
    370                 375                 380
Asn Trp Arg Glu Cys Lys Phe Val Glu Asp Glu Gly Asp Val Val Phe
385                 390                 395                 400
Tyr Lys His
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<210> 91
<211> 1428
<212> DNA
<213> Streptococcus mutans

<400> 91
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gaagtagatt atgtttatgc ttcagccaag aaagctcaac cagcttggcg atcactttca 180
tacatagaac gtgctgccta ccttcacaag gtagcagata ttttgatgcg tgataaagaa 240
aaaataggtg ctgttctttc caaagaggtt gctaaaggtt ataaatcagc agtcagcgaa 300
gttgttcgta ctgcagaaat cattaattat gcagctgaag aaggtcttcg tatggaaggt 360
gaagtccttg aaggcggcag ttttgaagca gccagcaaga aaaaaattgc cgttgttcgt 420
cgtgaaccag taggtcttgt attagctatt tcaccattta actaccctgt taacttggca 480
ggttcgaaaa ttgcaccggc tcttattgcg ggaaatgtta ttgcttttaa accaccgacg 540
caaggatcaa tctcagggct cttacttgct gaagcatttg ctgaagctgg acttcctgca 600
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caagccgtta actttatcaa ttttactggt tcaacaggaa ttggggaacg tattggcaaa 720
atggctggta tgcgtccgat tatgcttgaa ctcggtggaa aagattcagc catcgttctt 780
gaagatgcag accttgaatt gactgctaaa aatattattg caggtgcttt tggttattca 840
ggtcaacgct gtacagcagt taaacgtgtt cttgtgatgg aaagtgttgc tgatgaactg 900
gtcgaaaaaa tccgtgaaaa agttcttgca ttaacaattg gtaatccaga agacgatgca 960
gatattacac cgttgattga tacaaaatca gctgattatg tagaaggtct tattaatgat 1020
gccaatgata aaggagccac tgcccttact gaaatcaaac gtgaaggtaa tcttatctgt 1080
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tcggaatatg gacttcaggc ttctatcttt acaaatgatt tcccacgcgc ttttggtatt 1260
gctgagcagc ttgaagttgg tacagttcat atcaataata agacacagcg cggcacggac 1320
aacttcccat tcttaggggc taaaaaatca ggtgcaggta ttcaaggggt aaaatattct 1380
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<210> 92
<211> 475
<212> PRT
<213> Streptococcus mutans

<400> 92
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 1               5                  10                  15
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            20                  25                  30
Ser Val Pro Ala Met Ser Thr Glu Glu Val Asp Tyr Val Tyr Ala Ser
        35                  40                  45
Ala Lys Lys Ala Gln Pro Ala Trp Arg Ser Leu Ser Tyr Ile Glu Arg
    50                  55                  60
Ala Ala Tyr Leu His Lys Val Ala Asp Ile Leu Met Arg Asp Lys Glu
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65                  70                  75                  80
Lys Ile Gly Ala Val Leu Ser Lys Glu Val Ala Lys Gly Tyr Lys Ser
                85                  90                  95
Ala Val Ser Glu Val Val Arg Thr Ala Glu Ile Ile Asn Tyr Ala Ala
            100                 105                 110
Glu Glu Gly Leu Arg Met Glu Gly Glu Val Leu Glu Gly Gly Ser Phe
        115                 120                 125
Glu Ala Ala Ser Lys Lys Lys Ile Ala Val Val Arg Arg Glu Pro Val
    130                 135                 140
Gly Leu Val Leu Ala Ile Ser Pro Phe Asn Tyr Pro Val Asn Leu Ala
145                 150                 155                 160
Gly Ser Lys Ile Ala Pro Ala Leu Ile Ala Gly Asn Val Ile Ala Phe
                165                 170                 175
Lys Pro Pro Thr Gln Gly Ser Ile Ser Gly Leu Leu Leu Ala Glu Ala
            180                 185                 190
Phe Ala Glu Ala Gly Leu Pro Ala Gly Val Phe Asn Thr Ile Thr Gly
        195                 200                 205
Arg Gly Ser Glu Ile Gly Asp Tyr Ile Val Glu His Gln Ala Val Asn
    210                 215                 220
Phe Ile Asn Phe Thr Gly Ser Thr Gly Ile Gly Glu Arg Ile Gly Lys
225                 230                 235                 240
Met Ala Gly Met Arg Pro Ile Met Leu Glu Leu Gly Gly Lys Asp Ser
                245                 250                 255
Ala Ile Val Leu Glu Asp Ala Asp Leu Glu Leu Thr Ala Lys Asn Ile
            260                 265                 270
Ile Ala Gly Ala Phe Gly Tyr Ser Gly Gln Arg Cys Thr Ala Val Lys
        275                 280                 285
Arg Val Leu Val Met Glu Ser Val Ala Asp Glu Leu Val Glu Lys Ile
    290                 295                 300
Arg Glu Lys Val Leu Ala Leu Thr Ile Gly Asn Pro Glu Asp Asp Ala
305                 310                 315                 320
Asp Ile Thr Pro Leu Ile Asp Thr Lys Ser Ala Asp Tyr Val Glu Gly
                325                 330                 335
Leu Ile Asn Asp Ala Asn Asp Lys Gly Ala Thr Ala Leu Thr Glu Ile
            340                 345                 350
Lys Arg Glu Gly Asn Leu Ile Cys Pro Ile Leu Phe Asp Lys Val Thr
        355                 360                 365
Thr Asp Met Arg Leu Ala Trp Glu Glu Pro Phe Gly Pro Val Leu Pro
    370                 375                 380
Ile Ile Arg Val Thr Ser Val Glu Glu Ala Ile Glu Ile Ser Asn Lys
385                 390                 395                 400
Ser Glu Tyr Gly Leu Gln Ala Ser Ile Phe Thr Asn Asp Phe Pro Arg
                405                 410                 415
Ala Phe Gly Ile Ala Glu Gln Leu Glu Val Gly Thr Val His Ile Asn
            420                 425                 430
Asn Lys Thr Gln Arg Gly Thr Asp Asn Phe Pro Phe Leu Gly Ala Lys
        435                 440                 445
Lys Ser Gly Ala Gly Ile Gln Gly Val Lys Tyr Ser Ile Glu Ala Met
    450                 455                 460
Thr Thr Val Lys Ser Val Val Phe Asp Ile Lys
465                 470                 475
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<210> 93
<211> 1401
<212> DNA
<213> Aspergillus parasiticus

<400> 93

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tccctttcgt cgctggcgtc cgagtccgag aacaagggca agatgttgga cacctatgga 180
aatgagttca agatcccgga ctacaccatc aagcagatcc gtgacgccat ccccgctcac 240
tgttacgagc ggaaggctct caccagcttg tactatgtgt tccgtgacat agccatgctg 300
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gctcgcgttg ctttgtggtc cctttacacc gtcgtccagg gtctgatcgc tactggtgtc 420
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acccgcgagg agtatgcttc ccgcatcggc aagaccattc acgacctgaa cgaattgatg 660
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ccggccaagc tttccaaata g 1401

<210> 94
<211> 466
<212> PRT
<213> Aspergillus parasiticus

<400> 94

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Gly	Thr	Asp	Ser	Ser	Val	Pro	Ser	Val	Ser	Val	Ser	Pro	Phe	Asp	Ser
			20					25					30		
Pro	Arg	His	Ser	Pro	Ser	Ser	Thr	Ser	Leu	Ser	Ser	Leu	Ala	Ser	Glu
		35					40					45			
Ser	Glu	Asn	Lys	Gly	Lys	Met	Leu	Asp	Thr	Tyr	Gly	Asn	Glu	Phe	Lys
	50					55					60				
Ile	Pro	Asp	Tyr	Thr	Ile	Lys	Gln	Ile	Arg	Asp	Ala	Ile	Pro	Ala	His
65					70					75					80
Cys	Tyr	Glu	Arg	Lys	Ala	Leu	Thr	Ser	Leu	Tyr	Tyr	Val	Phe	Arg	Asp
				85					90					95	
Ile	Ala	Met	Leu	Gly	Ser	Ile	Phe	Tyr	Val	Phe	His	Asn	Tyr	Val	Thr
			100					105					110		
Pro	Glu	Thr	Val	Pro	Ser	Phe	Pro	Ala	Arg	Val	Ala	Leu	Trp	Ser	Leu
		115					120					125			
Tyr	Thr	Val	Val	Gln	Gly	Leu	Ile	Ala	Thr	Gly	Val	Trp	Val	Leu	Ala
	130					135					140				
His	Glu	Cys	Gly	His	Gln	Ala	Phe	Ser	Pro	Ser	Lys	Val	Leu	Asn	Asp
145					150					155					160
Thr	Val	Gly	Trp	Ile	Cys	His	Ser	Ala	Leu	Leu	Val	Pro	Tyr	Phe	Ser
				165					170					175	
Trp	Lys	Ile	Ser	His	Gly	Lys	His	His	Lys	Ala	Thr	Gly	Asn	Ile	Ala
			180					185					190		
Arg	Asp	Met	Val	Phe	Val	Pro	Lys	Thr	Arg	Glu	Glu	Tyr	Ala	Ser	Arg
		195					200					205			
Ile	Gly	Lys	Thr	Ile	His	Asp	Leu	Asn	Glu	Leu	Met	Glu	Glu	Thr	Pro
	210					215					220				

Ile Ala Thr Val Thr Asn Leu Ile Leu Gln Gln Leu Phe Gly Trp Pro
225 230 235 240
Met Tyr Leu Leu Thr Asn Val Thr Gly His Asn Asn His Glu Arg Gln
245 250 255
Pro Glu Gly Arg Gly Lys Gly Lys Arg Asn Gly Tyr Phe Gly Gly Val
260 265 270
Asn His Phe Asn Pro Ser Ser Pro Leu Tyr Glu Ala Lys Asp Ala Lys
275 280 285
Leu Ile Val Leu Ser Asp Leu Gly Leu Ala Ile Thr Gly Ser Val Leu
290 295 300
Tyr Tyr Ile Gly Ser Thr Tyr Gly Trp Leu Asn Leu Leu Val Trp Tyr
305 310 315 320
Gly Ile Pro Tyr Leu Trp Val Asn His Trp Leu Val Ala Ile Thr Tyr
325 330 335
Leu Gln His Thr Asp Pro Thr Leu Pro His Tyr Gln Pro Glu Val Trp
340 345 350
Asn Phe Ala Arg Gly Ala Ala Ala Thr Ile Asp Arg Asp Phe Gly Phe
355 360 365
Val Gly Arg His Ile Leu His Gly Ile Ile Glu Thr His Val Leu His
370 375 380
His Tyr Val Ser Thr Ile Pro Phe Tyr His Ala Asp Glu Ala Ser Glu
385 390 395 400
Ala Ile Gln Lys Val Met Gly Ser His Tyr Arg Thr Glu Ala His Thr
405 410 415
Gly Trp Thr Gly Phe Phe Lys Ala Leu Phe Thr Ser Ala Arg Val Cys
420 425 430
His Trp Val Glu Pro Thr Glu Gly Ala Lys Gly Glu Ser Glu Gly Val
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Leu Phe Tyr Arg Asn Thr Asn Gly Val Gly Val Pro Pro Ala Lys Leu
450 455 460
Ser Lys
465

<210> 95
<211> 1263
<212> DNA
<213> Pichia pastoris

<400> 95
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gacacctggg gtaacgtctt caaagtccct gattttacaa tcaagcaaat cttggatgct 180
attccaaagc actgctatga acgcaggcta accacgtcgt tctactacgt gttcagggac 240
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ttgtgggttt tggcccacga gtgcggtcat caagctttct ccgattatgg ttgggtcaac 420
gacaccgtgg gatggatttt gcattcttac ctgttagtcc catatttttc ttggaaatac 480
agtcatggta aacatcacaa ggctactggc cacttgacta gagacatggt gtttgtacct 540
gccacaaagg agaagttctt ggaaaagaga aacgccagca aacttggcga actgggagaa 600
gatgctccca ttttcacatt atatcagttg gtagcccaac aattgggagg ctggattttg 660
tatttgttca ccaacgttac tggtcaaccc taccccaaca cccctaaatg gatgcagaac 720
cactttgttc cctcatctcc aattttcgaa aaaaaggact actggtttat cattctgagt 780
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tggaacttat ttatttactg gttcctgcct tatgttctca ccaaccattg gctggtgttc 900
atcacattcc tgcaacactc tgatccaacg atgcctcact acgaggctga acaatggacg 960
tttgccagag gagctgccgc aaccatcgac cgtgaatttg gattcattgg acctttcttc 1020
tttcacgata tcatcgaaac tcacgtcttg catcactatg tgagtagaat tcctttctac 1080
aacgcccgag aggccagtga aggtattaag aaagttatgg gggagcatta tcgctacagt 1140

ggtgaaaaca tgtgggtctc tctttggaag agtggacgtt catgtcagtt tgttgatgga 1200
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tag 1263

<210> 96
<211> 420
<212> PRT
<213> Pichia pastoris

<400> 96

Met	Ser	Ala	Val	Thr	Val	Thr	Gly	Asn	Asn	Gly	Asp	Ala	Ser	Arg	Ser
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Asn	Thr	Thr	Thr	Thr	Thr	Lys	Arg	Thr	Gly	Asn	Val	Ser	Ser	Phe	Ser
			20					25					30		
Gln	Ser	Lys	Gly	Leu	Thr	Ala	Ile	Asp	Thr	Trp	Gly	Asn	Val	Phe	Lys
		35					40					45			
Val	Pro	Asp	Phe	Thr	Ile	Lys	Gln	Ile	Leu	Asp	Ala	Ile	Pro	Lys	His
	50					55					60				
Cys	Tyr	Glu	Arg	Arg	Leu	Thr	Thr	Ser	Phe	Tyr	Tyr	Val	Phe	Arg	Asp
65					70					75					80
Ile	Phe	Leu	Ile	Gly	Cys	Thr	Met	Phe	Met	Gly	Ser	Phe	Ile	Pro	Met
				85					90					95	
Ile	Glu	Asn	Val	Phe	Leu	Arg	Gly	Ala	Ala	Tyr	Ala	Ala	Leu	Val	Phe
			100					105					110		
Leu	Leu	Ser	Val	Glu	Tyr	Thr	Gly	Leu	Trp	Val	Leu	Ala	His	Glu	Cys
		115					120					125			
Gly	His	Gln	Ala	Phe	Ser	Asp	Tyr	Gly	Trp	Val	Asn	Asp	Thr	Val	Gly
	130					135					140				
Trp	Ile	Leu	His	Ser	Tyr	Leu	Leu	Val	Pro	Tyr	Phe	Ser	Trp	Lys	Tyr
145					150					155					160
Ser	His	Gly	Lys	His	His	Lys	Ala	Thr	Gly	His	Leu	Thr	Arg	Asp	Met
				165					170					175	
Val	Phe	Val	Pro	Ala	Thr	Lys	Glu	Lys	Phe	Leu	Glu	Lys	Arg	Asn	Ala
			180					185					190		
Ser	Lys	Leu	Gly	Glu	Leu	Gly	Glu	Asp	Ala	Pro	Ile	Phe	Thr	Leu	Tyr
		195					200					205			
Gln	Leu	Val	Ala	Gln	Gln	Leu	Gly	Gly	Trp	Ile	Leu	Tyr	Leu	Phe	Thr
	210					215					220				
Asn	Val	Thr	Gly	Gln	Pro	Tyr	Pro	Asn	Thr	Pro	Lys	Trp	Met	Gln	Asn
225					230					235					240
His	Phe	Val	Pro	Ser	Ser	Pro	Ile	Phe	Glu	Lys	Lys	Asp	Tyr	Trp	Phe
				245					250					255	
Ile	Ile	Leu	Ser	Asp	Leu	Gly	Ile	Leu	Ala	Gln	Leu	Met	Val	Leu	Tyr
			260					265					270		
Val	Trp	Arg	Gln	Gln	Met	Gly	Asn	Trp	Asn	Leu	Phe	Ile	Tyr	Trp	Phe
		275					280					285			
Leu	Pro	Tyr	Val	Leu	Thr	Asn	His	Trp	Leu	Val	Phe	Ile	Thr	Phe	Leu
	290					295					300				
Gln	His	Ser	Asp	Pro	Thr	Met	Pro	His	Tyr	Glu	Ala	Glu	Gln	Trp	Thr
305					310					315					320
Phe	Ala	Arg	Gly	Ala	Ala	Ala	Thr	Ile	Asp	Arg	Glu	Phe	Gly	Phe	Ile
				325					330					335	
Gly	Pro	Phe	Phe	Phe	His	Asp	Ile	Ile	Glu	Thr	His	Val	Leu	His	His
			340					345					350		
Tyr	Val	Ser	Arg	Ile	Pro	Phe	Tyr	Asn	Ala	Arg	Glu	Ala	Ser	Glu	Gly
		355					360					365			
Ile	Lys	Lys	Val	Met	Gly	Glu	His	Tyr	Arg	Tyr	Ser	Gly	Glu	Asn	Met
	370					375					380				
Trp	Val	Ser	Leu	Trp	Lys	Ser	Gly	Arg	Ser	Cys	Gln	Phe	Val	Asp	Gly

385 390 395 400
Glu Asn Gly Val Lys Met Tyr Arg Asn Ile Asn Asn Trp Gly Ile Gly
405 410 415
Thr Gly Glu Lys
420

<210> 97
<211> 3175
<212> DNA
<213> Marchantia polymorpha

<400> 97
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cagcgagcgc gagcgccaga gctcttgctc ccctcgctca tcgctcgcat tgccgcattt 240
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cacttgcttg tatgtccctg acgtgcttca aaattttgcg cggggaatca tcaatatact 3120
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<210> 98
<211> 481
<212> PRT
<213> Marchantia polymorpha

<400> 98

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Trp Ser Lys Trp Gly Thr Gly Ser Asn Leu Ser Phe Val Ser Arg Lys
            20                  25                  30
Glu Gln Gln Gln Gln Gln Gln Gln Ser Ser Pro Glu Ala Ser Thr Pro
        35                  40                  45
Ala Ala Gln Gln Glu Lys Ser Ile Ser Arg Glu Ser Ile Pro Glu Gly
    50                  55                  60
Phe Leu Thr Val Glu Glu Val Ser Lys His Asp Asn Pro Ser Asp Cys
65                  70                  75                  80
Trp Ile Val Ile Asn Asp Lys Val Tyr Asp Val Ser Ala Phe Gly Lys
                85                  90                  95
Thr His Pro Gly Gly Pro Val Ile Phe Thr Gln Ala Gly Arg Asp Ala
            100                 105                 110
Thr Asp Ser Phe Lys Val Phe His Ser Ala Lys Ala Trp Gln Phe Leu
        115                 120                 125
Gln Asp Leu Tyr Ile Gly Asp Leu Tyr Asn Ala Glu Pro Val Ser Glu
    130                 135                 140
Leu Val Lys Asp Tyr Arg Asp Leu Arg Thr Ala Phe Met Arg Ser Gln
145                 150                 155                 160
Leu Phe Lys Ser Ser Lys Met Tyr Tyr Val Thr Lys Cys Val Thr Asn
                165                 170                 175
Phe Ala Ile Leu Ala Ala Ser Leu Ala Val Ile Ala Trp Ser Gln Thr
            180                 185                 190
Tyr Leu Ala Val Leu Cys Ser Ser Phe Leu Leu Ala Leu Phe Trp Gln
        195                 200                 205
Gln Cys Gly Trp Leu Ser His Asp Phe Leu His His Gln Val Thr Glu
    210                 215                 220
Asn Arg Ser Leu Asn Thr Tyr Phe Gly Gly Leu Phe Trp Gly Asn Phe
225                 230                 235                 240
Ala Gln Gly Tyr Ser Val Gly Trp Trp Lys Thr Lys His Asn Val His
                245                 250                 255
His Ala Ala Thr Asn Glu Cys Asp Asp Lys Tyr Gln Pro Ile Asp Pro
            260                 265                 270
Asp Ile Asp Thr Val Pro Leu Leu Ala Trp Ser Lys Glu Ile Leu Ala
        275                 280                 285
Thr Val Asp Asp Gln Phe Phe Arg Ser Ile Ile Ser Val Gln His Leu
    290                 295                 300
Leu Phe Phe Pro Leu Leu Phe Leu Ala Arg Phe Ser Trp Leu His Ser
305                 310                 315                 320
Ser Trp Ala His Ala Ser Asn Phe Glu Met Pro Arg Tyr Met Arg Trp
                325                 330                 335
Ala Glu Lys Ala Ser Leu Leu Gly His Tyr Gly Ala Ser Ile Gly Ala
            340                 345                 350
Ala Phe Tyr Ile Leu Pro Ile Pro Gln Ala Ile Cys Trp Leu Phe Leu
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        355                 360                 365
Ser Gln Leu Phe Cys Gly Ala Leu Leu Ser Ile Val Phe Val Ile Ser
    370                 375                 380
His Asn Gly Met Asp Val Tyr Asn Asp Pro Arg Asp Phe Val Thr Ala
385                 390                 395                 400
Gln Val Thr Ser Thr Arg Asn Ile Glu Gly Asn Phe Phe Asn Asp Trp
                405                 410                 415
Phe Thr Gly Gly Leu Asn Arg Gln Ile Glu His His Leu Phe Pro Ser
            420                 425                 430
Leu Pro Arg His Asn Leu Ala Lys Val Ala Pro His Val Lys Ala Leu
        435                 440                 445
Cys Ala Lys His Gly Leu His Tyr Glu Glu Leu Ser Leu Gly Thr Gly
    450                 455                 460
Val Cys Arg Val Phe Asn Arg Leu Val Glu Val Ala Tyr Ala Ala Lys
465                 470                 475                 480
Val
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<210> 99
<211> 1335
<212> DNA
<213> Cyprinus carpio

<400> 99

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tacacctggg aggaggtgca gaaacacacc aagtttggag atcagtggat cgaggttgaa 120
aggaaggttt ataatgtgag ccagtgggtg aagagacacc ccggaggagt gaggatcctc 180
ggacactatg ctggagaaga tgccacggag gcgtttactg catttcatcc agaccttccg 240
ctggtgagaa aatacatgaa gccgctgtta atcggggagc ttgaggcgtc tgaacccagt 300
caagaccggc agaaaaatgc tgctcttgtg gaggacttcc gagcccttcg tgagcgtctg 360
gaggctgagg ggtgtttcaa aacccagccg ctgtttctca tcttacatct gagtcacatc 420
ctgctcctgg aggccatcgc tctgatgatg gtgtggtacc tgggaaccgg ctggatcaac 480
acggccatcg tcgctgtttt actggccact gcacagtcac aggctgaatg gttgcagcac 540
gacttcggtc atctgtccgt gtttaaaacc tctcgatgga atcacctggt gcacaaattt 600
gtcgtcggac acattaaggg agcgtctgcg ggtcggtgga accatcgcca cttccagcat 660
cacgctaaac cgaacgtgtt caaaaaggac ccggacgtca acatgctcaa tgcgtttgtg 720
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cctcacgttc gagagttatg tgccaaatac ggaatcaagt accaagagaa gaccttgcag 1260
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<210> 100
<211> 444
<212> PRT
<213> Cyprinus carpio

<400> 100

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Arg Phe Gly Thr Tyr Thr Trp Glu Glu Val Gln Lys His Thr Lys Phe
            20                  25                  30
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Gly Asp Gln Trp Ile Glu Val Glu Arg Lys Val Tyr Asn Val Ser Gln
35 40 45
Trp Val Lys Arg His Pro Gly Gly Val Arg Ile Leu Gly His Tyr Ala
50 55 60
Gly Glu Asp Ala Thr Glu Ala Phe Thr Ala Phe His Pro Asp Leu Pro
65 70 75 80
Leu Val Arg Lys Tyr Met Lys Pro Leu Leu Ile Gly Glu Leu Glu Ala
85 90 95
Ser Glu Pro Ser Gln Asp Arg Gln Lys Asn Ala Ala Leu Val Glu Asp
100 105 110
Phe Arg Ala Leu Arg Glu Arg Leu Glu Ala Glu Gly Cys Phe Lys Thr
115 120 125
Gln Pro Leu Phe Leu Ile Leu His Leu Ser His Ile Leu Leu Leu Glu
130 135 140
Ala Ile Ala Leu Met Met Val Trp Tyr Leu Gly Thr Gly Trp Ile Asn
145 150 155 160
Thr Ala Ile Val Ala Val Leu Leu Ala Thr Ala Gln Ser Gln Ala Glu
165 170 175
Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Lys Thr Ser Arg
180 185 190
Trp Asn His Leu Val His Lys Phe Val Val Gly His Ile Lys Gly Ala
195 200 205
Ser Ala Gly Arg Trp Asn His Arg His Phe Gln His His Ala Lys Pro
210 215 220
Asn Val Phe Lys Lys Asp Pro Asp Val Asn Met Leu Asn Ala Phe Val
225 230 235 240
Ala Gly Lys Val Gln Pro Val Glu Tyr Gly Val Lys Lys Ile Lys His
245 250 255
Leu Pro Tyr Asn His Gln His Lys Tyr Phe Phe Phe Ile Gly Pro Pro
260 265 270
Leu Leu Ile Pro Val Tyr Phe Gln Phe Gln Ile Phe His Asn Met Ile
275 280 285
Ala His Gly Leu Trp Val Asp Leu Ala Trp Cys Ile Ser Tyr Tyr Val
290 295 300
Arg Tyr Phe Leu Cys Tyr Thr Gln Tyr Tyr Gly Val Phe Trp Ala Val
305 310 315 320
Ile Leu Phe Asn Phe Val Arg Phe Leu Lys Ser His Trp Phe Val Trp
325 330 335
Val Thr Gln Met Ser His Ile Pro Met Gln Ile Asp Tyr Glu Lys His
340 345 350
Gln Asp Arg Leu Ser Met Gln Leu Val Ala Thr Cys Asn Ile Glu Gln
355 360 365
Ser Ser Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
370 375 380
His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr Trp Arg Ala Ala
385 390 395 400
Pro His Val Arg Glu Leu Cys Ala Lys Tyr Gly Ile Lys Tyr Gln Glu
405 410 415
Lys Thr Leu Gln Gly Ala Phe Ala Asp Val Val Arg Ser Leu Glu Lys
420 425 430
Ser Gly Glu Ile Trp Leu Asp Ala Tyr Leu Asn Glu
435 440

<210> 101
<211> 894
<212> DNA
<213> Salmo salar

<400> 101
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atgtacctgc tgatcgtatg gctggggccc aagtacatga gacacagaca gccggtgtct 180
tgccggggtc tcctgttggt ctacaatctg ggcctcacga tcttgtcctt ctatatgttc 240
tatgagatgg tgtctgctgt gtggcacggg gattataact tctattgcca agacacacac 300
agtgcaggag aaaccgatac caagatcata aatgtgctgt ggtggtacta cttctccaaa 360
gctcatagag ttttatggac accttcttct tcatccctac ggaagaacaa ccatcagatc 420
acgtttctgc acatctacca ccatgctagc atgctcaaca tctggtggtt cgtcatgaac 480
tgggtgccct gtggtcactc ctactttggt gcctccctga acagcttcat ccatgtcctg 540
atgtactctt actatgggct ctctgctgtc ccggccttgc ggccctatct atggtggaag 600
aagtatacat cacacaagca cagcttgatt cagttctttt tgaccatgtc ccagacgata 660
tgtgcagtca tttggccatg tggtttcccc agagggtggc tgtatttcca gatattctat 720
gtcgtcacac ttattgccct tttctcaaac ttctacattc agacttacaa gaaacacctt 780
gtttcacaaa agaaggagtg tcatcagaat ggctctgttg cttcattgaa tggccatgtg 840
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<210> 102
<211> 297
<212> PRT
<213> Salmo salar

<400> 102
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Gly Pro Arg Asp Glu Arg Val Gln Gly Trp Leu Leu Leu Asp Asn Tyr
20 25 30
Pro Pro Thr Phe Ala Leu Thr Val Met Tyr Leu Leu Ile Val Trp Leu
35 40 45
Gly Pro Lys Tyr Met Arg His Arg Gln Pro Val Ser Cys Arg Gly Leu
50 55 60
Leu Leu Val Tyr Asn Leu Gly Leu Thr Ile Leu Ser Phe Tyr Met Phe
65 70 75 80
Tyr Glu Met Val Ser Ala Val Trp His Gly Asp Tyr Asn Phe Tyr Cys
85 90 95
Gln Asp Thr His Ser Ala Gly Glu Thr Asp Thr Lys Ile Ile Asn Val
100 105 110
Leu Trp Trp Tyr Tyr Phe Ser Lys Ala His Arg Val Leu Trp Thr Pro
115 120 125
Ser Ser Ser Ser Leu Arg Lys Asn Asn His Gln Ile Thr Phe Leu His
130 135 140
Ile Tyr His His Ala Ser Met Leu Asn Ile Trp Trp Phe Val Met Asn
145 150 155 160
Trp Val Pro Cys Gly His Ser Tyr Phe Gly Ala Ser Leu Asn Ser Phe
165 170 175
Ile His Val Leu Met Tyr Ser Tyr Tyr Gly Leu Ser Ala Val Pro Ala
180 185 190
Leu Arg Pro Tyr Leu Trp Trp Lys Lys Tyr Thr Ser His Lys His Ser
195 200 205
Leu Ile Gln Phe Phe Leu Thr Met Ser Gln Thr Ile Cys Ala Val Ile
210 215 220
Trp Pro Cys Gly Phe Pro Arg Gly Trp Leu Tyr Phe Gln Ile Phe Tyr
225 230 235 240
Val Val Thr Leu Ile Ala Leu Phe Ser Asn Phe Tyr Ile Gln Thr Tyr
245 250 255
Lys Lys His Leu Val Ser Gln Lys Lys Glu Cys His Gln Asn Gly Ser
260 265 270
Val Ala Ser Leu Asn Gly His Val Asn Gly Val Thr Pro Thr Glu Thr
275 280 285

Ile Thr His Arg Lys Val Arg Gly Asp
290 295

<210> 103
<211> 873
<212> DNA
<213> Marchantia polymorpha

<400> 103
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gatagcccga cgccgatcgt tcttgggttg tcgtcctact tgacattcgt gtttctcggg 180
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gaagttcaaa tgggccacct gctctacatt ttctacatgt caaagtacat cgagtttatg 420
gacacggtca ttatgatttt gaagcgcaac acgcgccaga tcactgtgtt gcatgtgtac 480
caccacgcat ccatctcctt catctggtgg atcatcgcct accatgctcc tggcggtgaa 540
gcttatttct ctgccgcatt gaactccgga gtacatgtgc tcatgtacct ctactacctt 600
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tacttgacac agctgcagat gttccagttt gtccttaaca tgattcaggc ttactacgat 720
attaagaaca actcgcctta cccacaattt ttgatccaga ttttgttcta ctacatgatc 780
tcgcttttag cgctatttgg aaacttttac gttcacaaat acgtatcagc gcccgcaaaa 840
cctgcgaaga tcaagagcaa aaaggcagaa taa 873

<210> 104
<211> 290
<212> PRT
<213> Marchantia polymorpha

<400> 104
Met Glu Ala Tyr Glu Met Val Asp Ser Phe Val Ser Lys Thr Val Phe
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Glu Thr Leu Gln Arg Leu Arg Gly Gly Val Val Leu Thr Glu Ser Ala
20 25 30
Ile Thr Lys Gly Leu Pro Cys Val Asp Ser Pro Thr Pro Ile Val Leu
35 40 45
Gly Leu Ser Ser Tyr Leu Thr Phe Val Phe Leu Gly Leu Ile Val Ile
50 55 60
Lys Ser Leu Asp Leu Lys Pro Arg Ser Lys Glu Pro Ala Ile Leu Asn
65 70 75 80
Leu Phe Val Ile Phe His Asn Phe Val Cys Phe Ala Leu Ser Leu Tyr
85 90 95
Met Cys Val Gly Ile Val Arg Gln Ala Ile Leu Asn Arg Tyr Ser Leu
100 105 110
Trp Gly Asn Ala Tyr Asn Pro Lys Glu Val Gln Met Gly His Leu Leu
115 120 125
Tyr Ile Phe Tyr Met Ser Lys Tyr Ile Glu Phe Met Asp Thr Val Ile
130 135 140
Met Ile Leu Lys Arg Asn Thr Arg Gln Ile Thr Val Leu His Val Tyr
145 150 155 160
His His Ala Ser Ile Ser Phe Ile Trp Trp Ile Ile Ala Tyr His Ala
165 170 175
Pro Gly Gly Glu Ala Tyr Phe Ser Ala Ala Leu Asn Ser Gly Val His
180 185 190
Val Leu Met Tyr Leu Tyr Tyr Leu Leu Ala Ala Thr Leu Gly Lys Asn
195 200 205
Glu Lys Ala Arg Arg Lys Tyr Leu Trp Trp Gly Lys Tyr Leu Thr Gln

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    210                 215                 220
Leu Gln Met Phe Gln Phe Val Leu Asn Met Ile Gln Ala Tyr Tyr Asp
225                 230                 235                 240
Ile Lys Asn Asn Ser Pro Tyr Pro Gln Phe Leu Ile Gln Ile Leu Phe
                245                 250                 255
Tyr Tyr Met Ile Ser Leu Leu Ala Leu Phe Gly Asn Phe Tyr Val His
            260                 265                 270
Lys Tyr Val Ser Ala Pro Ala Lys Pro Ala Lys Ile Lys Ser Lys Lys
        275                 280                 285
Ala Glu
    290
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<210> 105
<211> 1365
<212> DNA
<213> Salmo salar

<400> 105

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cccgatggag ggcaaggtgg cagtgcagtc tacacctggg aagaggtcca gaggcactcc 120
cacagaagcg accagtggtt ggtcatcgac aggaaggtct ataatattac ccagtgggca 180
aagagacacc cgggtggcat cagggtcatc agtcactttg ctggagaaga tgccacggaa 240
gcattttccg cattccatct tgatgctaat tttgtcagga agtttctgaa gccgttgctg 300
attggagagc tggcaccgac agagcccagc caggaccatg ggaaaaatgc agctctggtg 360
caggacttcc aggccttgcg tgaccatgtg gagagggagg gtctcctccg tgcccgcctc 420
ctgttcttca gcctctacct gggccacatc ctgctactag aggccctggc tttgggcctg 480
ctctgggtct gggggaccag ctggagcctc acactgctct gttccctcat gctggccacg 540
tctcaggccc aggctggctg gctgcagcat gactacggcc acctgtcagt ctgcaagaaa 600
tccagctgga accacaaact gcacaagttt gtcattggac acctaaaggg tgcctctgct 660
aactggtgga accatcgtca cttccagcac cacgctaagc ccaacgtgtt tcgtaaagat 720
cctgatatca actcactgcc tgtcttcgtc ctgggagaca cacagcctgt agagtatggt 780
ataaagaagt tgaagtacat gccctaccat caccaacacc agtacttctt cctcattgga 840
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cgggactggg tggatctggc gtggtcgatg agtttctacc ttcgcttctt ctgctgttac 960
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agacaccagg actggctcac catgcagttg agcgctactt gcaacattga acagtcaacc 1140
ttcaacgact ggttcagtgg acacctcaac tttcagattg aacaccatct gtttcctacc 1200
atgccccgtc ataactacca cctggtggct cctctggtgc gtactttgtg tgagaaacat 1260
ggagttccct atcaggtcaa gactttgcag aaaggcatga ctgatgttgt caggtcactg 1320
aagaagtcag gggatctgtg gctggatgca tatctccata aatga                 1365
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<210> 106
<211> 454
<212> PRT
<213> Salmo salar

<400> 106

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Met Gly Gly Gly Gly Gln Gln Thr Glu Ser Ser Glu Pro Ala Lys Gly
 1               5                  10                  15
Asp Gly Leu Glu Pro Asp Gly Gly Gln Gly Gly Ser Ala Val Tyr Thr
            20                  25                  30
Trp Glu Glu Val Gln Arg His Ser His Arg Ser Asp Gln Trp Leu Val
        35                  40                  45
Ile Asp Arg Lys Val Tyr Asn Ile Thr Gln Trp Ala Lys Arg His Pro
    50                  55                  60
Gly Gly Ile Arg Val Ile Ser His Phe Ala Gly Glu Asp Ala Thr Glu
65                  70                  75                  80
```

Ala Phe Ser Ala Phe His Leu Asp Ala Asn Phe Val Arg Lys Phe Leu
85 90 95
Lys Pro Leu Leu Ile Gly Glu Leu Ala Pro Thr Glu Pro Ser Gln Asp
100 105 110
His Gly Lys Asn Ala Ala Leu Val Gln Asp Phe Gln Ala Leu Arg Asp
115 120 125
His Val Glu Arg Glu Gly Leu Leu Arg Ala Arg Leu Leu Phe Phe Ser
130 135 140
Leu Tyr Leu Gly His Ile Leu Leu Leu Glu Ala Leu Ala Leu Gly Leu
145 150 155 160
Leu Trp Val Trp Gly Thr Ser Trp Ser Leu Thr Leu Leu Cys Ser Leu
165 170 175
Met Leu Ala Thr Ser Gln Ala Gln Ala Gly Trp Leu Gln His Asp Tyr
180 185 190
Gly His Leu Ser Val Cys Lys Lys Ser Ser Trp Asn His Lys Leu His
195 200 205
Lys Phe Val Ile Gly His Leu Lys Gly Ala Ser Ala Asn Trp Trp Asn
210 215 220
His Arg His Phe Gln His His Ala Lys Pro Asn Val Phe Arg Lys Asp
225 230 235 240
Pro Asp Ile Asn Ser Leu Pro Val Phe Val Leu Gly Asp Thr Gln Pro
245 250 255
Val Glu Tyr Gly Ile Lys Lys Leu Lys Tyr Met Pro Tyr His His Gln
260 265 270
His Gln Tyr Phe Phe Leu Ile Gly Pro Pro Leu Ile Val Pro Val Phe
275 280 285
Phe Asn Ile Gln Ile Phe Arg Thr Met Phe Ser Gln Arg Asp Trp Val
290 295 300
Asp Leu Ala Trp Ser Met Ser Phe Tyr Leu Arg Phe Phe Cys Cys Tyr
305 310 315 320
Tyr Pro Phe Phe Gly Phe Phe Gly Ser Val Ala Leu Ile Ser Phe Val
325 330 335
Arg Phe Leu Glu Ser His Trp Phe Val Trp Val Thr Gln Met Asn His
340 345 350
Leu Pro Met Glu Met Asp His Glu Arg His Gln Asp Trp Leu Thr Met
355 360 365
Gln Leu Ser Ala Thr Cys Asn Ile Glu Gln Ser Thr Phe Asn Asp Trp
370 375 380
Phe Ser Gly His Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Thr
385 390 395 400
Met Pro Arg His Asn Tyr His Leu Val Ala Pro Leu Val Arg Thr Leu
405 410 415
Cys Glu Lys His Gly Val Pro Tyr Gln Val Lys Thr Leu Gln Lys Gly
420 425 430
Met Thr Asp Val Val Arg Ser Leu Lys Lys Ser Gly Asp Leu Trp Leu
435 440 445
Asp Ala Tyr Leu His Lys
450

<210> 107
<211> 1455
<212> DNA
<213> Marchantia polymorpha

<400> 107
atgccgccac acgcccctga ctccacaggt cttgggcccg aagttttccg cctgcctgat 60
gacgcgatcc cggcccagga tcgcagatct acacagaaga aatactcgct ttcagacgtc 120
agcaagcaca acactccgaa tgattgctgg ctcgtaattt gggggaaggt gtacgatgtt 180

acttcgtggg ttaaggtcca tccaggtgga agtctcatct ttgtgaaggc gggacaggat 240
tcaacacaac tctttgattc ttatcacccc ctctatgtca gaaagctact tgcacagttc 300
tgcattggtg aactccaaac gagtgcggga gatgagaagt tcaagtcttc aacgttggag 360
tatgctggtg aagaacatga agtattttac cacactctca agcagcgcgt ggaaacgtac 420
ttccgcaagc agaagataaa tcctcgatac catccgcaaa tgcttgtgaa gtcagccgtg 480
atcattggaa cccttcttct ctgttactat tttggcttct tctggtctca aaatgtactc 540
ctctcgatgt ttctggcaag catcatgggg ttctgcactg cggaggtggg catgtccatc 600
atgcacgatg gtaaccacgg atcgtacaca caatctacct tgcttggtta cgtcatgggc 660
gccactcttg atctggtggg agctagcagt ttcatgtgga ggcagcagca tgtggccggg 720
caccactcgt tcaccaacat cgaccattac gatccagaca ttcgtgtgaa ggatcctgat 780
ttacgacggg ttacttctca acaaccccga agatggtttc acgagtatca gcatatctac 840
ttaggagtac tctatggcgt tcttgcctta aaaagtgtgt tgattgatga tttcagcgcc 900
ttcttcagtg gtgctatcgg cccagtaaag atagctcaaa tgacaccact cgagatgggc 960
gtcttctggg gagggaaggt tgtgtacgca ctgtacatgt ttttgctccc tatgatgtat 1020
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tggactcttg ccctcttctt tcaagtagca cacgttgtcg acgatgcagt atttcccgtt 1140
gcggaaacag atggtggaaa agcaaagatt ccttctggtt gggcagaaat gcaggtcaga 1200
accactacca atttcagctc acgatcaatg ttctggacac atattagtgg cggtctgaac 1260
catcagatcg agcaccatct tttcccgggt gtctgtcatg ttcactaccc aagcatacag 1320
ccaatcgtga aggctacctg tgacgagttc aacgtgcctt atacttccta ccccactttc 1380
tgggcggccc ttagggcaca ttttcaacat ctgaaaaacg tcggactaca agatggacta 1440
cgactggatg gctga 1455

<210> 108
<211> 484
<212> PRT
<213> Marchantia polymorpha

<400> 108

Met	Pro	Pro	His	Ala	Pro	Asp	Ser	Thr	Gly	Leu	Gly	Pro	Glu	Val	Phe
1				5					10					15	
Arg	Leu	Pro	Asp	Asp	Ala	Ile	Pro	Ala	Gln	Asp	Arg	Arg	Ser	Thr	Gln
			20					25					30		
Lys	Lys	Tyr	Ser	Leu	Ser	Asp	Val	Ser	Lys	His	Asn	Thr	Pro	Asn	Asp
		35					40					45			
Cys	Trp	Leu	Val	Ile	Trp	Gly	Lys	Val	Tyr	Asp	Val	Thr	Ser	Trp	Val
	50					55					60				
Lys	Val	His	Pro	Gly	Gly	Ser	Leu	Ile	Phe	Val	Lys	Ala	Gly	Gln	Asp
65					70					75					80
Ser	Thr	Gln	Leu	Phe	Asp	Ser	Tyr	His	Pro	Leu	Tyr	Val	Arg	Lys	Leu
			85					90					95		
Leu	Ala	Gln	Phe	Cys	Ile	Gly	Glu	Leu	Gln	Thr	Ser	Ala	Gly	Asp	Glu
		100					105					110			
Lys	Phe	Lys	Ser	Ser	Thr	Leu	Glu	Tyr	Ala	Gly	Glu	Glu	His	Glu	Val
	115					120					125				
Phe	Tyr	His	Thr	Leu	Lys	Gln	Arg	Val	Glu	Thr	Tyr	Phe	Arg	Lys	Gln
130					135					140					
Lys	Ile	Asn	Pro	Arg	Tyr	His	Pro	Gln	Met	Leu	Val	Lys	Ser	Ala	Val
145					150					155					160
Ile	Ile	Gly	Thr	Leu	Leu	Leu	Cys	Tyr	Tyr	Phe	Gly	Phe	Phe	Trp	Ser
			165					170					175		
Gln	Asn	Val	Leu	Leu	Ser	Met	Phe	Leu	Ala	Ser	Ile	Met	Gly	Phe	Cys
		180					185					190			
Thr	Ala	Glu	Val	Gly	Met	Ser	Ile	Met	His	Asp	Gly	Asn	His	Gly	Ser
	195					200					205				
Tyr	Thr	Gln	Ser	Thr	Leu	Leu	Gly	Tyr	Val	Met	Gly	Ala	Thr	Leu	Asp
210					215					220					
Leu	Val	Gly	Ala	Ser	Ser	Phe	Met	Trp	Arg	Gln	Gln	His	Val	Ala	Gly
225					230					235					240

His His Ser Phe Thr Asn Ile Asp His Tyr Asp Pro Asp Ile Arg Val
245 250 255
Lys Asp Pro Asp Leu Arg Arg Val Thr Ser Gln Gln Pro Arg Arg Trp
260 265 270
Phe His Glu Tyr Gln His Ile Tyr Leu Gly Val Leu Tyr Gly Val Leu
275 280 285
Ala Leu Lys Ser Val Leu Ile Asp Asp Phe Ser Ala Phe Phe Ser Gly
290 295 300
Ala Ile Gly Pro Val Lys Ile Ala Gln Met Thr Pro Leu Glu Met Gly
305 310 315 320
Val Phe Trp Gly Gly Lys Val Val Tyr Ala Leu Tyr Met Phe Leu Leu
325 330 335
Pro Met Met Tyr Gly Gln Tyr Asn Ile Leu Thr Phe Ile Gly Leu Tyr
340 345 350
Ile Leu Ser Gln Leu Val Ala Gly Trp Thr Leu Ala Leu Phe Phe Gln
355 360 365
Val Ala His Val Val Asp Asp Ala Val Phe Pro Val Ala Glu Thr Asp
370 375 380
Gly Gly Lys Ala Lys Ile Pro Ser Gly Trp Ala Glu Met Gln Val Arg
385 390 395 400
Thr Thr Thr Asn Phe Ser Ser Arg Ser Met Phe Trp Thr His Ile Ser
405 410 415
Gly Gly Leu Asn His Gln Ile Glu His His Leu Phe Pro Gly Val Cys
420 425 430
His Val His Tyr Pro Ser Ile Gln Pro Ile Val Lys Ala Thr Cys Asp
435 440 445
Glu Phe Asn Val Pro Tyr Thr Ser Tyr Pro Thr Phe Trp Ala Ala Leu
450 455 460
Arg Ala His Phe Gln His Leu Lys Asn Val Gly Leu Gln Asp Gly Leu
465 470 475 480
Arg Leu Asp Gly

<210> 109
<211> 1338
<212> DNA
<213> Pavlova lutheri

<400> 109
atgccgcctt cggccgcgag cgagggcggc gtggcggagc tgcgcgcggc ggaggtcgcc 60
tcgtacacgc gcaaggcggt ggatgagcgc cccgacctca ccatcgtcgg cgatgccgtc 120
tacgacgcca aggccttccg tgacgagcac ccgggcggcg cccactttgt gagcctcttt 180
ggcgggcgcg acgcgaccga ggcgttcatg gagtaccacc ggcgggcgtg gcccaaggcg 240
cggatgagca agttcttcgt gggctcgctc gacgcctccg agaagccgac gcaggccgac 300
agtgcctacc tccggctgtg cgcggaggtg aacgccttgc tgccaaaggg gagcggcggc 360
tttgcgccgc cctcctattg gctcaaggcg gcggcgctgg tggtggccgc cgtgtcgatt 420
gaggggtata tgctgctgcg cggcaagacg ctcctcctct ccgtctttct cggcctcgtc 480
tttgcgtgga tcggtctcaa catccagcac gacgcgaacc acggcgcgct ctcgcgccac 540
tcggtgatca actactgcct tgggtacgcg caggactgga tcggcggcaa catggtgctc 600
tggctgcagg agcacgtggt gatgcaccac ctgcacacca acgacgtcga cgccgacccg 660
gaccagaagg cgcacggcgt gctgcggctc aagccaacgg acggctggat gccgtggcat 720
gcgctccaac agctttacat tctgcccggc gaggcgatgt acgcgtttaa gctgctcttc 780
ctcgacgcgc tcgagctgct cgcgtggcga tgggagggcg agaagatctc gcccctcgcg 840
cgcgccctgt ttgcaccagc ggtggcgtgc aagcttggct tctgggcgcg cttcgtcgcg 900
ctgccgctct ggctgcagcc gacggtgcac acggcgctgt gcatctgcgc gacggtgtgc 960
acgggctcct tctacctcgc cttcttcttc ttcatctcgc acaactttga cggcgtgggt 1020
agtgtgggcc ccaagggcag cttgccgcgc tctgcaacct tcgtgcagcg gcaggtcgag 1080
acgagttcga atgtgggcgg ctactggctt ggcgtgctca atggagggct caacttccag 1140

atcgagcacc atcttttccc gcggctgcac cattcgtact acgcgcagat tgccccagtg 1200
gtgcgcacgc acatcgagaa gctcggcttc aagtacaggc acttccccac ggtgggctcc 1260
aacttgtcgt ccatgctgca gcacatgggc aagatgggca ctcgcccagg agctgagaag 1320
ggcggcaagg ccgagtga 1338

<210> 110
<211> 445
<212> PRT
<213> Pavlova lutheri

<400> 110

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Met Pro Pro Ser Ala Ala Ser Glu Gly Gly Val Ala Glu Leu Arg Ala
 1               5                  10                  15
Ala Glu Val Ala Ser Tyr Thr Arg Lys Ala Val Asp Glu Arg Pro Asp
            20                  25                  30
Leu Thr Ile Val Gly Asp Ala Val Tyr Asp Ala Lys Ala Phe Arg Asp
        35                  40                  45
Glu His Pro Gly Gly Ala His Phe Val Ser Leu Phe Gly Gly Arg Asp
    50                  55                  60
Ala Thr Glu Ala Phe Met Glu Tyr His Arg Arg Ala Trp Pro Lys Ala
65                  70                  75                  80
Arg Met Ser Lys Phe Phe Val Gly Ser Leu Asp Ala Ser Glu Lys Pro
                85                  90                  95
Thr Gln Ala Asp Ser Ala Tyr Leu Arg Leu Cys Ala Glu Val Asn Ala
            100                 105                 110
Leu Leu Pro Lys Gly Ser Gly Gly Phe Ala Pro Pro Ser Tyr Trp Leu
        115                 120                 125
Lys Ala Ala Ala Leu Val Val Ala Ala Val Ser Ile Glu Gly Tyr Met
    130                 135                 140
Leu Leu Arg Gly Lys Thr Leu Leu Leu Ser Val Phe Leu Gly Leu Val
145                 150                 155                 160
Phe Ala Trp Ile Gly Leu Asn Ile Gln His Asp Ala Asn His Gly Ala
                165                 170                 175
Leu Ser Arg His Ser Val Ile Asn Tyr Cys Leu Gly Tyr Ala Gln Asp
            180                 185                 190
Trp Ile Gly Gly Asn Met Val Leu Trp Leu Gln Glu His Val Val Met
        195                 200                 205
His His Leu His Thr Asn Asp Val Asp Ala Asp Pro Asp Gln Lys Ala
    210                 215                 220
His Gly Val Leu Arg Leu Lys Pro Thr Asp Gly Trp Met Pro Trp His
225                 230                 235                 240
Ala Leu Gln Gln Leu Tyr Ile Leu Pro Gly Glu Ala Met Tyr Ala Phe
                245                 250                 255
Lys Leu Leu Phe Leu Asp Ala Leu Glu Leu Leu Ala Trp Arg Trp Glu
            260                 265                 270
Gly Glu Lys Ile Ser Pro Leu Ala Arg Ala Leu Phe Ala Pro Ala Val
        275                 280                 285
Ala Cys Lys Leu Gly Phe Trp Ala Arg Phe Val Ala Leu Pro Leu Trp
    290                 295                 300
Leu Gln Pro Thr Val His Thr Ala Leu Cys Ile Cys Ala Thr Val Cys
305                 310                 315                 320
Thr Gly Ser Phe Tyr Leu Ala Phe Phe Phe Phe Ile Ser His Asn Phe
                325                 330                 335
Asp Gly Val Gly Ser Val Gly Pro Lys Gly Ser Leu Pro Arg Ser Ala
            340                 345                 350
Thr Phe Val Gln Arg Gln Val Glu Thr Ser Ser Asn Val Gly Gly Tyr
        355                 360                 365
Trp Leu Gly Val Leu Asn Gly Gly Leu Asn Phe Gln Ile Glu His His
    370                 375                 380
```

```
Leu Phe Pro Arg Leu His His Ser Tyr Tyr Ala Gln Ile Ala Pro Val
385                 390                 395                 400
Val Arg Thr His Ile Glu Lys Leu Gly Phe Lys Tyr Arg His Phe Pro
                405                 410                 415
Thr Val Gly Ser Asn Leu Ser Ser Met Leu Gln His Met Gly Lys Met
            420                 425                 430
Gly Thr Arg Pro Gly Ala Glu Lys Gly Gly Lys Ala Glu
        435                 440                 445
```

<210> 111
<211> 1077
<212> DNA
<213> Saproleigna diclina

<400> 111
atgactgagg ataagacgaa ggtcgagttc ccgacgctca cggagctcaa gcactcgatc 60
ccgaacgcgt gctttgagtc gaacctcggc ctctcgctct actacacggc ccgcgcgatc 120
ttcaacgcgt cggcctcggc ggcgctgctc tacgcggcgc gctcgacgcc gttcattgcc 180
gataacgttc tgctccacgc gctcgtttgc gccacctaca tctacgtgca gggcgtcatc 240
ttctggggct tcttcacggt cggccacgac tgcggccact cggccttctc gcgctaccac 300
agcgtcaact ttatcatcgg ctgcatcatg cactctgcga ttttgacgcc gttcgagagc 360
tggcgcgtga cgcaccgcca ccaccacaag aacacgggca acattgataa ggacgagatc 420
ttttacccgc accggtcggt caaggacctc caggacgtgc gccaatgggt ctacacgctc 480
ggcggtgcgt ggtttgtcta cttgaaggtc gggtatgccc cgcgcacgat gagccacttt 540
gacccgtggg acccgctcct ccttcgccgc gcgtcggccg tcatcgtgtc gctcggcgtc 600
tgggccgcct tcttcgccgc gtacgcgtac ctcacatact cgctcggctt tgccgtcatg 660
ggcctctact actatgcgcc gctctttgtc tttgcttcgt tcctcgtcat tacgaccttc 720
ttgcaccaca acgacgaagc gacgccgtgg tacggcgact cggagtggac gtacgtcaag 780
ggcaacctct cgagcgtcga ccgctcgtac ggcgcgttcg tggacaacct gagccaccac 840
attggcacgc accaggtcca ccacttgttc ccgatcattc cgcactacaa gctcaacgaa 900
gccaccaagc actttgcggc cgcgtacccg cacctcgtgc gcaggaacga cgagcccatc 960
atcacggcct tcttcaagac cgcgcacctc tttgtcaact acggcgctgt gcccgagacg 1020
gcgcagatct tcacgctcaa agagtcggcc gcggccgcca aggccaagtc ggactaa 1077

<210> 112
<211> 358
<212> PRT
<213> Saproleigna diclina

<400> 112
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Met Thr Glu Asp Lys Thr Lys Val Glu Phe Pro Thr Leu Thr Glu Leu
 1               5                  10                  15
Lys His Ser Ile Pro Asn Ala Cys Phe Glu Ser Asn Leu Gly Leu Ser
            20                  25                  30
Leu Tyr Tyr Thr Ala Arg Ala Ile Phe Asn Ala Ser Ala Ser Ala Ala
        35                  40                  45
Leu Leu Tyr Ala Ala Arg Ser Thr Pro Phe Ile Ala Asp Asn Val Leu
    50                  55                  60
Leu His Ala Leu Val Cys Ala Thr Tyr Ile Tyr Val Gln Gly Val Ile
65                  70                  75                  80
Phe Trp Gly Phe Phe Thr Val Gly His Asp Cys Gly His Ser Ala Phe
                85                  90                  95
Ser Arg Tyr His Ser Val Asn Phe Ile Ile Gly Cys Ile Met His Ser
            100                 105                 110
Ala Ile Leu Thr Pro Phe Glu Ser Trp Arg Val Thr His Arg His His
        115                 120                 125
His Lys Asn Thr Gly Asn Ile Asp Lys Asp Glu Ile Phe Tyr Pro His
    130                 135                 140
```

Arg Ser Val Lys Asp Leu Gln Asp Val Arg Gln Trp Val Tyr Thr Leu
145 150 155 160
Gly Gly Ala Trp Phe Val Tyr Leu Lys Val Gly Tyr Ala Pro Arg Thr
165 170 175
Met Ser His Phe Asp Pro Trp Asp Pro Leu Leu Leu Arg Arg Ala Ser
180 185 190
Ala Val Ile Val Ser Leu Gly Val Trp Ala Ala Phe Phe Ala Ala Tyr
195 200 205
Ala Tyr Leu Thr Tyr Ser Leu Gly Phe Ala Val Met Gly Leu Tyr Tyr
210 215 220
Tyr Ala Pro Leu Phe Val Phe Ala Ser Phe Leu Val Ile Thr Thr Phe
225 230 235 240
Leu His His Asn Asp Glu Ala Thr Pro Trp Tyr Gly Asp Ser Glu Trp
245 250 255
Thr Tyr Val Lys Gly Asn Leu Ser Ser Val Asp Arg Ser Tyr Gly Ala
260 265 270
Phe Val Asp Asn Leu Ser His His Ile Gly Thr His Gln Val His His
275 280 285
Leu Phe Pro Ile Ile Pro His Tyr Lys Leu Asn Glu Ala Thr Lys His
290 295 300
Phe Ala Ala Ala Tyr Pro His Leu Val Arg Arg Asn Asp Glu Pro Ile
305 310 315 320
Ile Thr Ala Phe Phe Lys Thr Ala His Leu Phe Val Asn Tyr Gly Ala
325 330 335
Val Pro Glu Thr Ala Gln Ile Phe Thr Leu Lys Glu Ser Ala Ala Ala
340 345 350
Ala Lys Ala Lys Ser Asp
355

<210> 113
<211> 1251
<212> DNA
<213> Saccharomyces kluyveri

<400> 113
atgtctgcag ttacagtcac agggtccgac ccaaagaacc gtggttcctc ctctaatacc 60
gagcaagaag ttccaaaagt tgcaattgac accaatggta atgttttcag cgtaccagat 120
tttaccatca aggatatttt gggcgccatt ccccacgaat gttacgaaag aagactagca 180
acatcgttat actatgtttt tagagacatc ttctgcatgc taacaaccgg ttaccttaca 240
cacaaaatct tatatccatt gctgatctca tacacttcta actcaataat caagtttacc 300
ttctgggctt tgtacacata cgtccaaggt ttgtttggta ctggtatctg ggtgttggcc 360
cacgaatgtg gccatcaagc cttctcagac tatggtattg tcaacgattt tgttggctgg 420
actctacact cttacttgat ggtaccatat ttttcgtgga agtattccca tggtaagcat 480
cacaaggcca ccggtcacat gactagagac atggtttttg ttcctgccac aaaggaggaa 540
tttaagaaaa gcagaaactt tttcggaaat ttggcagaat actccgagga ttccccatta 600
agaactttgt acgaattgct ggtacaacaa ctaggaggtt ggattgcata tctttttgtc 660
aacgttactg gtcaaccgta tccagatgtt ccttcctgga aatggaacca cttctggcta 720
acttctccat tatttgaaca aagggatgct ttgtacattt ttttgagtga tctaggtatc 780
ttgacccaag gcattgtttt gaccttgtgg tacaagaagt ttggtggctg gtctctgttc 840
atcaattggt ttgttccata catttgggtt aaccactggt tggtttttat cactttttg 900
caacacaccg acccaactat gccccattac aatgctgagg aatggacttt tgccaagggt 960
gctgccgcca ccattgatag aaaattcggg tttattggtc ctcacatttt ccatgacatt 1020
attgaaaccc atgtgctaca ccactactgt agcagaattc cattctataa cgctcgtcca 1080
gcaagcgagg ctattaagaa agtgatgggc aagcattata gatctagtga cgaaaacatg 1140
tggaagtcct tatggaagtc ttttagatct tgtcagtatg ttgatggaga caatggtgtt 1200
ttaatgttca gaaacatcaa caactgtggt gttggcgccg ctgagaaatg a 1251

<210> 114

<211> 416
<212> PRT
<213> Saccharomyces kluyveri

<400> 114

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Met Ser Ala Val Thr Val Thr Gly Ser Asp Pro Lys Asn Arg Gly Ser
 1               5                  10                  15
Ser Ser Asn Thr Glu Gln Glu Val Pro Lys Val Ala Ile Asp Thr Asn
            20                  25                  30
Gly Asn Val Phe Ser Val Pro Asp Phe Thr Ile Lys Asp Ile Leu Gly
        35                  40                  45
Ala Ile Pro His Glu Cys Tyr Glu Arg Arg Leu Ala Thr Ser Leu Tyr
    50                  55                  60
Tyr Val Phe Arg Asp Ile Phe Cys Met Leu Thr Thr Gly Tyr Leu Thr
65                  70                  75                  80
His Lys Ile Leu Tyr Pro Leu Leu Ile Ser Tyr Thr Ser Asn Ser Ile
                85                  90                  95
Ile Lys Phe Thr Phe Trp Ala Leu Tyr Thr Tyr Val Gln Gly Leu Phe
            100                 105                 110
Gly Thr Gly Ile Trp Val Leu Ala His Glu Cys Gly His Gln Ala Phe
        115                 120                 125
Ser Asp Tyr Gly Ile Val Asn Asp Phe Val Gly Trp Thr Leu His Ser
    130                 135                 140
Tyr Leu Met Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Gly Lys His
145                 150                 155                 160
His Lys Ala Thr Gly His Met Thr Arg Asp Met Val Phe Val Pro Ala
                165                 170                 175
Thr Lys Glu Glu Phe Lys Lys Ser Arg Asn Phe Phe Gly Asn Leu Ala
            180                 185                 190
Glu Tyr Ser Glu Asp Ser Pro Leu Arg Thr Leu Tyr Glu Leu Leu Val
        195                 200                 205
Gln Gln Leu Gly Gly Trp Ile Ala Tyr Leu Phe Val Asn Val Thr Gly
    210                 215                 220
Gln Pro Tyr Pro Asp Val Pro Ser Trp Lys Trp Asn His Phe Trp Leu
225                 230                 235                 240
Thr Ser Pro Leu Phe Glu Gln Arg Asp Ala Leu Tyr Ile Phe Leu Ser
                245                 250                 255
Asp Leu Gly Ile Leu Thr Gln Gly Ile Val Leu Thr Leu Trp Tyr Lys
            260                 265                 270
Lys Phe Gly Gly Trp Ser Leu Phe Ile Asn Trp Phe Val Pro Tyr Ile
        275                 280                 285
Trp Val Asn His Trp Leu Val Phe Ile Thr Phe Leu Gln His Thr Asp
    290                 295                 300
Pro Thr Met Pro His Tyr Asn Ala Glu Glu Trp Thr Phe Ala Lys Gly
305                 310                 315                 320
Ala Ala Ala Thr Ile Asp Arg Lys Phe Gly Phe Ile Gly Pro His Ile
                325                 330                 335
Phe His Asp Ile Ile Glu Thr His Val Leu His His Tyr Cys Ser Arg
            340                 345                 350
Ile Pro Phe Tyr Asn Ala Arg Pro Ala Ser Glu Ala Ile Lys Lys Val
        355                 360                 365
Met Gly Lys His Tyr Arg Ser Ser Asp Glu Asn Met Trp Lys Ser Leu
    370                 375                 380
Trp Lys Ser Phe Arg Ser Cys Gln Tyr Val Asp Gly Asp Asn Gly Val
385                 390                 395                 400
Leu Met Phe Arg Asn Ile Asn Asn Cys Gly Val Gly Ala Ala Glu Lys
                405                 410                 415
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